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GROWTH

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SYNERGISTIC EFFECT OF THYROXIN AND ESTRADIOL VALERATE ON THE INHIBITION OF LINEAR GROWTH IN THE MOUSE FEMUR

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(Received May 28, 1959)

In juvenile mice, estrogen inhibits the proliferation and hypertrophy of cartilage cells in the epiphyseal plate (Silberberg and Silberberg, '46) and resorption of cartilage (Silberberg and Silberberg, '41). Furthermore, estrogen appears to stimulate osteogenesis and inhibit bone resorption (Gardner and Pfeiffer, '43). Long bones of newborn mice injected with estrogen apparently ossify endosteally within 10 days (Urist et al, '50). Estradiol causes an increase in the number of ossification centers present in female rats, but not in male rats of the same juvenile age (Talbot, '39). However, Noback et al. ('49) concluded that estradiol did not induce the early appearance of ossification centers in either sex. Thus, estrogen appears to inhibit linear growth when given in large doses by inducing sclerosis and hyalinization of the epiphyseal plate (Gardner and Pfeiffer, '43; Silberberg and Silberberg, '56).

Thyroxin, given in large doses, stimulates the conversion of proliferating cartilage cells into hypertrophic cells, and accelerates the resorption of cartilage and metaphyseal trabeculae in young mice (Silberberg and Silberberg, '46). As a corollary, thyroxin accelerates the appearance of ossification centers in both sexes, while thiouracil delays them in young rats (Noback et al., '49; Asling et al., '51, p. 146). The main influence of thyroxin appears to be that of skeletal maturation and only secondarily that of influencing skeletal growth (Asling et al., '51).

Thyroxin, when given in combination with estradiol, appears to counteract partly the estradiol-induced inhibition of proliferation and resorption of cartilage in young mice (Silberberg and Silberberg, '46). However, Baisset et al. ('54) concluded that the two hormones when

given together induce an additive effect on the narrowing of the epiphyseal cartilage in adult rats. Both groups of investigators agree that the degeneration of cartilage is intense under these conditions. With the combined treatment, trabeculae are less abundant than with estrogen treatment alone (Silberberg and Silberberg, '46; Baisset et al., '54). Growth studies on the combined effects of thyroxin and estrogen in young mice appear to be lacking.

The purposes of this investigation are to determine: (1) whether or not thyroxin or estrogen will induce premature maturation of bone and inhibition of bone growth in neonatal mice, (2) if estrogen might induce a differential sex response to early maturation of bone in young mice, and (3) if the combination of thyroxin and estrogen will bring about an additive effect in inhibiting linear growth in young mice.

Materials and Methods

Eighty-nine $C_{57}B1$ mice were injected subcutaneously beginning when one day old. One μg of thyroxin in .025cm³ of 0.9% of saline was injected three times weekly for two weeks. Five μg of estradiol-17-β-n-valerate (hereafter called estradiol valerate) in .05 cm³ of sesame oil were injected once weekly for two weeks. In preliminary experiments, higher dosages of thyroxin were toxic to the neonatal mice.

The mice were divided into four groups: Group I received the saline and sesame oil carriers; Group II received a total of 7μg of thyroxin; Group III received a total of 15 μg of estradiol valerate; Group IV received similar amounts of the hormone and steroid. The numbers and distribution in each group are tabulated in Table I.

TABLE I
Mean Femur Lengths of 21-Day Old Mice

	Treatment	Sex	No. of Mice	Femur lengths in mm. ± Standard Deviation
I	Control	♂	11	10.1 ± .5
II	Thyroxin	♂	17	9.7 ± .1
III	Estradiol Valerate	♂	11	9.4 ± .6
IV	Estradiol Valerate + Thyroxin	♂	11	8.9 ± .5
I	Control	♀	9	9.9 ± .6
II	Thyroxin	♀	9	9.5 ± .3
III	Estradiol Valerate	♀	10	9.5 ± .6
IV	Estradiol Valerate + Thyroxin	♀	11	8.7 ± .7
	Total		89	

The mice were sacrificed at three weeks of age. The right femur was fixed in modified Bouin's fixative (Suzuki, '58a), embedded by Peterfi's double embedding method (Carleton and Drury, '57, p. 50), and stained with Harris' hematoxylin and eosin. The left femurs were removed, dried, cleaned and measured by a previously described method (Suzuki, '58b). The statistical analysis was based on Fisher's unpaired *t* test for significance.

Results

The following descriptions will be limited to the distal femoral epiphyses, metaphyses and diaphyses. No sex differences were observed in the histology of the epiphyses and metaphyses in any group.

Group I: Carrier-treated controls, (Figure 1). The epiphysis had thin bony trabeculae. The cartilaginous epiphysis was wide and continuous with bands of hyaline cartilage on the anterior and posterior condylar surfaces of the femur. The cartilage on the condylar surfaces was composed of roundish cartilage cells and lacunae, two or three layers deep. The cartilaginous plate had regular rows of proliferating cartilage composed of 10-12 cells. The nuclei were somewhat flattened, often with two or three in a lacuna. There was a fair amount of matrix between the cartilage rows. The hypertrophied cartilage cells numbered between 3-4 per row. The metaphysis had short, straight, thin trabeculae. Many pyramidal osteoblasts and small blood vessels were present in this region.

Group II: Thyroxin-treated mice, (Figure 2). The epiphyseal and metaphyseal trabeculae were similar to the carrier-treated controls. Thyroxin induced early maturation of the cartilaginous tissues. In contrast to the controls, cartilage cells were replaced by bone in the condylar regions. The cartilaginous plates appeared to be similar to the controls; the proliferating cells ranging from 9-15 per row, while hypertrophied cells numbered from 2-4 per row. However, the plate was narrower because the cartilage nuclei and lacunae were more flattened and there was a decreased amount of matrix in the thyroxin treated mice. The blood vessels in the metaphyses were larger and more numerous than those of the controls.

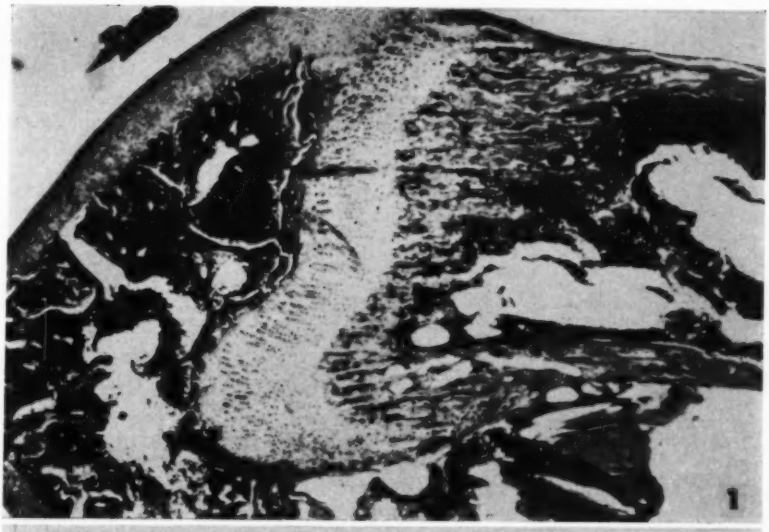
Group III: Estradiol valerate-treated mice, (Figure 3). The epiphyseal trabeculae were somewhat thicker and more numerous than those in the two previous groups. The epiphyseal plate and condylar

FIGURE 1

Distal portion of a femur from a normal 21-day old mouse. The epiphyseal plate is composed of a wide band of cartilage cells which are continuous with the cartilage cells in the condylar regions. The metaphysis has many short straight trabeculae. (× 44).

FIGURE 2

Distal portion of a femur from a 21-day old mouse treated with a total of 7 μgms of thyroxin. The epiphyseal plate is narrower because the cells are smaller and more closely packed. The condylar regions have been replaced by bone. The metaphysis appears similar to the normal control mouse. (× 44)



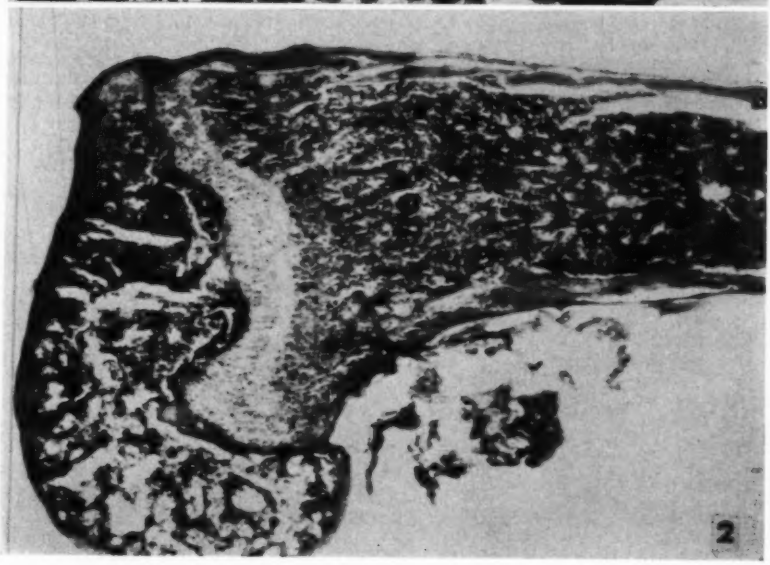
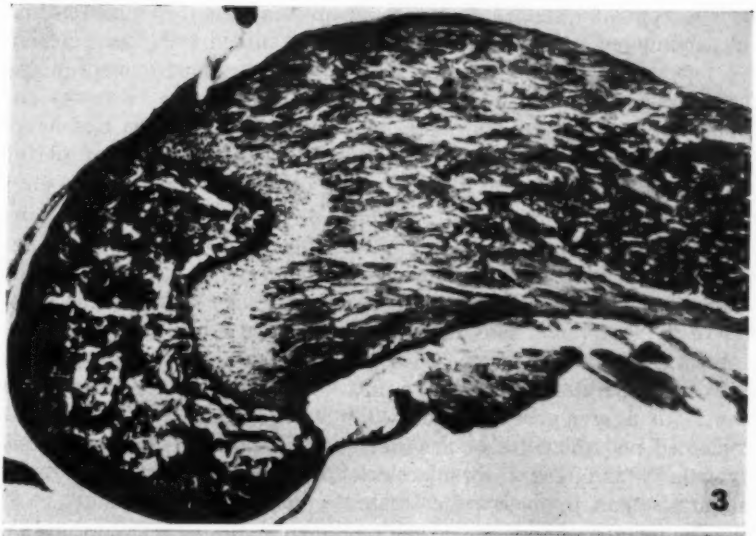


FIGURE 3

Distal portion of a femur from a 21-day old mouse treated with a total of 15 μgms of estradiol valerate. The epiphyseal plate is narrowed because the cells are smaller and more closely packed. The metaphysis and distal part of the diaphysis have been replaced by coalesced trabeculae of bone. (× 44)

FIGURE 4

Distal portion of a femur from a 21-day old mouse treated with a total of 7 μgms of thyroxin and 15 μgms of estradiol valerate. The epiphyseal plate is narrowed, with small cartilaginous cells. The epiphyseal marrow, in addition to the metaphysis and distal part of the diaphysis, has been replaced by coalesced trabeculae. Note also that the trabeculated bone extends more proximally into the diaphysis in comparison to the estrogen-treated mouse. (× 44)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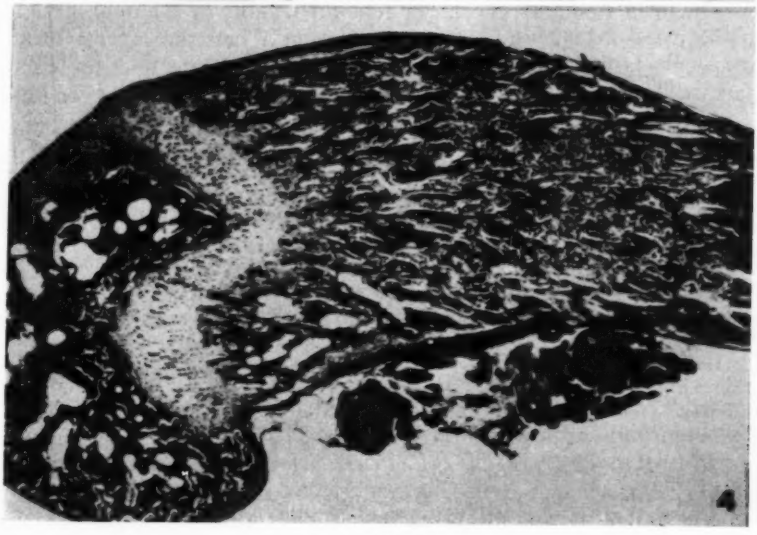
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regions appeared similar to the thyroxin-treated mice. Proliferating cells numbered 9-15 per row, while hypertrophied cells ranged from 3-4 per row. Likewise the size and number of blood vessels in the metaphysis appeared similar to group II. Unlike the two previous groups the metaphysis and distal portion of the diaphysis had many coalesced, irregularly shaped trabeculae, which replaced part of the distal femoral marrow cavity.

Group IV: Thyroxin plus estradiol valerate-treated group, (Figure 4). The cartilaginous epiphysis and condylar regions appeared similar to groups given either thyroxin or estradiol valerate separately. The number of proliferating cells ranged from 8-13 per row, and the number of hypertrophied cells ranged from 3-4 per row. The metaphysis appeared similar to group III. The combination of thyroxin and estradiol valerate intensified ossification in the epiphysis and diaphysis to a greater extent than did estrogen per se. Thus, many coalesced and thick trabeculae were in the epiphysis, and coalesced, irregularly arranged trabeculae extended more proximally into the diaphysis than in those mice treated with estradiol valerate per se.

Growth of femurs. The mean femoral lengths in the four groups are shown on Table I. The control group I had the longest mean femoral length, while the thyroxin, estradiol valerate and the combined treatment group had progessively shorter mean femoral lengths.

Table II summarizes the probabilities of differences in mean femoral lengths in the four groups. Femurs of thyroxin-treated mice of both sexes were not significantly shorter than those of the controls. Estradiol valerate-treated male mice had significantly shorter femurs,

TABLE II
PROBABILITIES OF DIFFERENCES IN FEMUR LENGTHS

Group	Sex Compared	Thyroxin	Estradiol Valerate	Estradiol Valerate + Thyroxin
Control	♂	N.S.*	$P < .01$	$P < .001$
Thyroxin	♂	——	N.S.	$P < .001$
Estradiol Valerate	♂	——	——	$P < .05$
Control	♀	N.S.	N.S.	$P < .001$
Thyroxin	♀	——	N.S.	$P < .01$
Estradiol Valerate	♀	——	——	$P < .02$

*N.S. Not significant.

while femurs of female mice in the same groups were not significantly different in length. With the combined treatment, femurs were significantly shorter than those from the other groups in both sexes.

Discussion

The epiphyseal plates were narrowed and the condylar cartilage cells were prematurely maturated in the hyperthyroid neonatal mice. Although the number of cartilage cells per row were similar to the controls, the cells and lacunae in the hyperthyroid mice were smaller with less matrix around them. The results reported herein agree with other studies in which experimentally induced hyperthyroidism accelerated the rate of cartilage maturation and replacement by bone (Asling et al., '51; Noback et al., '49), followed by premature closure or narrowing of the epiphyses and the consequent cessation or inhibition of growth (Baisset et al., '54; McLean and Urist, '55, p. 86; Smith and McLean, '38). In the present experiments, there was little or no resorption of metaphyseal trabeculae, although thyroxin enhanced resorption of trabeculae in young mice treated for a minimum of a month (Silberberg and Silberberg, '46). The apparent contradiction in fact may be due to differences in age of the mice used, and/or amounts of thyroxin administered. Due to a relatively high growth potential of bone in unweaned mice, thyroxin may not be able to reverse mineral deposition in the metaphyses. The newborn mice received smaller amounts of thyroxin per body weight in comparison to those given to two months old mice used by the Silberbergs, because administration of higher doses of thyroxin proved toxic to neonatal mice.

Linear growth of the femurs was not modified with the amounts of thyroxin injected into the mice. Thyroidectomized rats show retardation in growth and delay in the formation of ossification centers; these resume with replacement therapy (Asling et al., '51). As early as 1894, Hofmeister and von Eiselberg (quoted by Sissons, '56) independently reported that the surgical removal of the thyroid gland inhibits growth. In consideration of a temporal relationship as a necessary consideration for determining the action of thyroxin, rats kept hyperthyroid from 4 to 12 weeks seemed to have a temporary increase in growth rate, followed by a retardation of growth (Smith and McLean, '38).

The epiphyseal plates and condylar regions of mice treated with estradiol valerate appeared similar to those treated with thyroxin. The metaphyses and distal portions of the diaphyses had a number of trabeculae, which were coalesced in irregular arrangements, essentially substantiating the findings of Urist et al. ('50), who obtained endosteal response within 10 days after injecting estradiol benzoate into newborn mice. In consideration of a possible mechanism of estrogenic action upon bone, experiments by Zachariae ('58) indicate that estrogen induces the large production of acid sulfomucopolysaccharides in the uterus, oviducts and vagina as evidenced by increased uptake of radiosulfate. One of the functions of polysaccharides may be to link together collagen fibrils (Eastoe, '56). In addition, Rubin and Howard ('50) suggested that mucopolysaccharides, specifically chondroitin sulfate, might aid in the fixation of calcium in mineral deposition of bone. Finally, Moss ('58) reported that osteogenesis was induced after chondroitin sulfate was impregnated in Gelfoam and placed intracerebrally into rat brains. Therefore, estrogen may indirectly aid in hyperossification through the formation of large amounts of chondroitin sulfate.

Femurs from male mice treated with estradiol valerate were significantly shorter than the controls. Adult male mice are apparently more responsive to endosteal bone changes (Urist et al., '48), although Gardner and Pfeiffer ('38) felt that male mice were less responsive because of the antagonistic action of androgens. Although it is not clear why estradiol valerate inhibited growth of femurs only in male neonatal mice, it is possible that androgens may synergize the growth inhibiting effects of estrogens when present in certain ratios, as suggested by Jun and Peczenik (quoted by Allen et al., '39, p. 508). Femurs from untreated males were longer, though not significantly in comparison to those of females, while femurs from estrogen-treated males were shorter than those of females so treated. Thus, it is possible that endogenous estrogens may have somewhat inhibited linear growth so that the further addition of exogenous estrogens may have been without effect on growth.

The present investigation has substantiated the fact that thyroxin and estrogen when given independently induced the premature maturation of cartilage. When the hormone and steroid were given simultaneously they acted in concert by augmenting hyperossification

and by inhibiting linear femoral growth more significantly than did estrogen per se. These results are not in concordance with the results of Silberberg and Silberberg ('46), who demonstrated in mice that thyroxin counteracted the excess bone deposition induced by estrogen. The dissimilarities of experimental design may explain, at least in part, the disparities in results. It is possible that thyroxin, with the dosages used, conditioned osseous tissues to respond more readily to estradiol valerate. Perhaps, estrogen, in the presence of a definite ratio of thyroxin, may act together in the hyperstimulation of the formation of organic matrix. This is suggested even though thyroxin is known to inhibit the incorporation of radiosulfate into sulfomucopolysaccharides (Asboe-Hansen, '59). Experiments are now being designed along these lines.

Summary

Thyroxin and estradiol valerate, when given independently, induced premature maturation of cartilage. While thyroxin did not induce resorption of bony metaphyseal trabeculae, estradiol valerate inhibited bone resorption and stimulated endochondral and endosteal ossification in the distal femoral metaphysis. Thyroxin, per se, did not inhibit bone growth, while estradiol valerate inhibited bone growth only in the males so treated. Estradiol valerate and thyroxin acted in concert to produce further augmentation of hyperossification and inhibition of growth. Some of the possible mechanisms and further problems on the subject were considered.

Acknowledgments

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Growth, 1959, **23**, 303-311.

DIFFERENTIAL EFFECTS OF VARIOUS ANDROGENS ON SOMATIC GROWTH AND ON GONOPODIAL DEVELOPMENT IN THE CYPRINODONT: LEBISTES RETICULATUS.

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Received September 14, 1959

Cyprinodonts present a certain number of secondary sexual characters, one of which concerns the size of individuals. In *Lebistes reticulatus,* adult males measure about 17 mm in total length, while adult females may reach 30 to 40 mm in length (measured from the tip of the snout to the base of the caudal fin).

The action of androgens on somatic growth was studied by several authors: Eversole (1939-42), Gallien (1946), Regnier (1941) in *Lebistes reticulatus;* Cohen (1946) in *Platypoecilus maculatus* and Hamon (1945) in *Gambusia affinis.* All these authors have arrived at the same conclusion, that androgenic substances induce an inhibiting action on somatic growth. This inhibiting action was interpreted as merely a masculinising one.

Induction of gonopodium-like structures in treated females, by administration of androgenic steroids was demonstrated in several species of Cyprinodonts: Baldwin and Goldin (1941) in *Xiphophorus helleri;* Eversole (1939), Regnier (1941) and Gallien (1948) in *Lebistes reticulatus;* Lepori (1941) and Hamon (1945) in *Gambusia affinis;* Laskowski (1953) in *Platypoecilus* (*maculatus* & *variatus*) and Cumming (1943) in *Mollienisia latipinna.*

Quantitative studies were undertaken by Sangster (1948) in order to determine the optimum dose of ethynyl testosterone for the induction of a typical gonopodial structure, in females of *Xiphophorus helleri.* The action of androgens on the regenerating capacity of anal fins was studied by Grobstein (1940) in *Platypoecilus maculatus* and by Hopper (1949) in *Lebistes reticulatus.*

The following experiment was undertaken for the following two purposes:

(1) Determination of the nature of the inhibiting action of androgens on somatic growth.

(2) Analysis of the differential effects of equivalent doses of various androgens of gonopodial development.

Experiment

106 animals were treated from birth for a period of 6 months. Treated animals were divided into 4 groups

38 received 0.03 mg of pregneninolone 3 times a week,
31 received 0.03 mg of methyl testosterone 3 times a week,
23 received 0.03 mg of testosterone propionate 3 times a week,
14 received 0.03 mg of transdehydroandrosterone 3 times a week.

The aquarium water was renewed every time a new dose of androgenic substance was added. A fifth group of control animals (20) was raised in an identical manner except for the androgen treatment. Measurements were carried out with an ocular micrometer.

Results

I. *Action of androgens on total body length.*

All treated animals, regardless of sex, showed considerable growth inhibition. At the end of the experiment, individuals treated with methyl testosterone were the smallest in size, those treated with transdehydroandrosterone were the greatest.

From figure 1 we can remark that:

(a) In each group of treated animals, the average size of males and females was approximately the same;

(b) The average total length of all treated males and females, is less than that of normal control males.

These two points probably can be explained by an indirect inhibiting action of androgens on somatic growth, through the pituitary gland. The fact that the average total length of females treated with transdehydroandrosterone is clearly higher than that of treated males, is probably due to an antagonistic effect of estrogens secreted by the ovaries. Ovaries of treated females in this group (and only in this one) are perfectly normal.

II. *Action of androgens on gonopodial development.*

(A) *Action of pregneninolone*

All treated animals, regardless of sex, show a transformation of the anal fin into a more or less typical gonopodium. Gonopodia of treated animals are shorter than those of the controls. In both sexes,

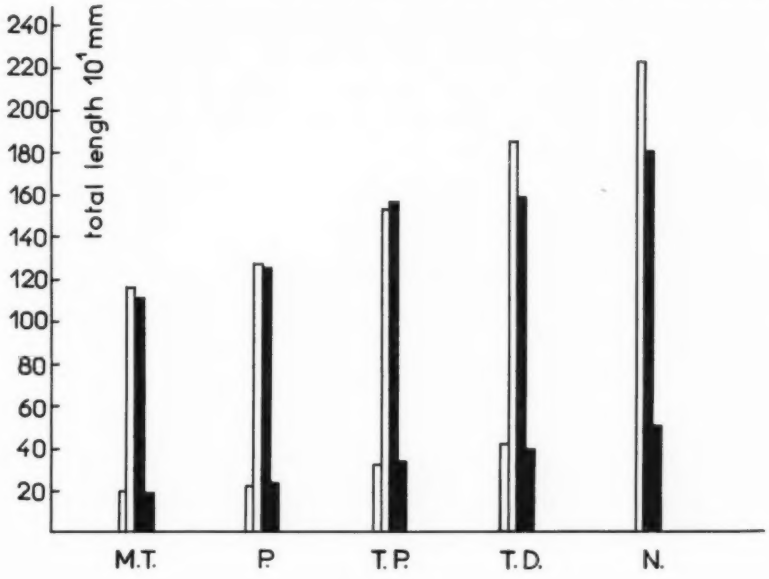
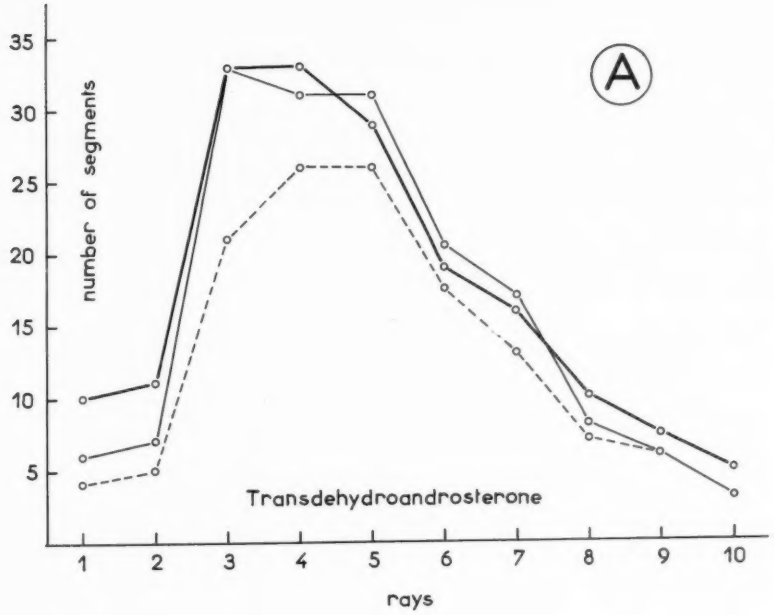


FIGURE 1

Diagram showing the mean length of treated animals as compared with those of control ones. Black column on the right: mean length of gonopodia in normal and treated ♂. Black column on the left: mean length of normal and treated males. White column on the left: mean length of gonopodia in treated females. White column on the right: mean body length of normal and treated females. N: control; M.T., P., T.P. and T.D.: animals treated with methyltestosterone, pregneninolone, testosterone propionate and transdehydroandrosterone.

the ratio total length/gonopodial length is equal to 5.4 in average, while in control males this ratio is equal to 3.6 in average. The number of segments is reduced in all rays of the anal fin. From this point of view, females present a slightly more typical gonopodium. While in normal adult males, the average number of segments on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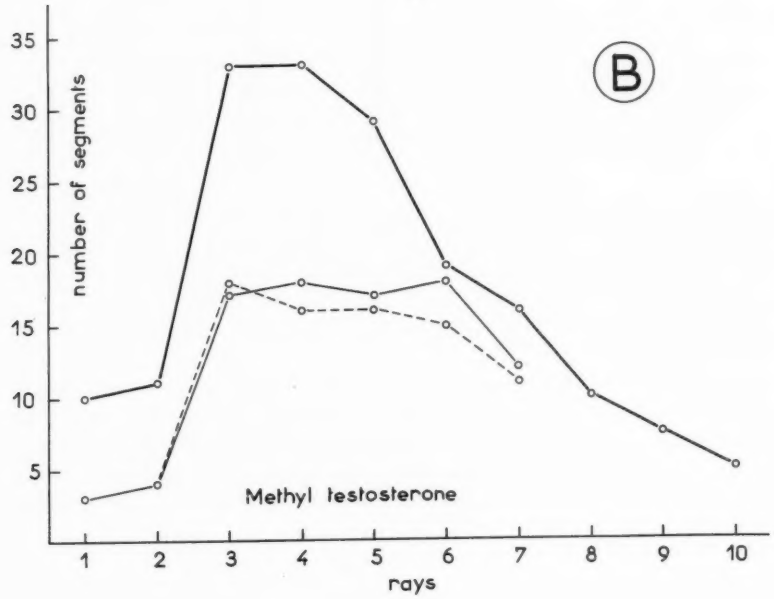
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rays

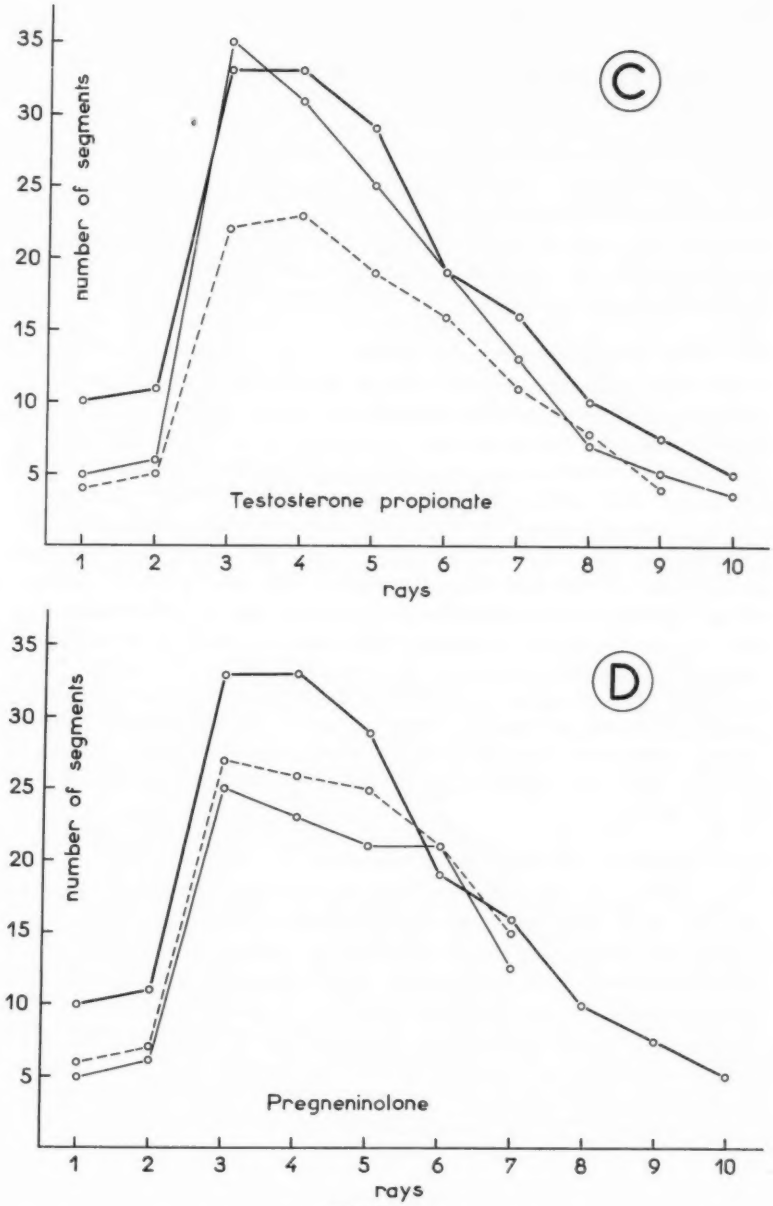

FIGURE 2

Graphs representing the mean number of segments in each fin ray, in treated females (dotted line) and in treated males (fine full line) as compared with those found in normal males (thick full line).

the third ray is about 33, their average number is respectively 25 and 27 in treated males and females. Specific characters of the gonopodium are present, i.e. ventral and dorsal spines, as well as the terminal hook, but there are not as many spines in treated fish as in the control males. The average number of ventral spines is about 15. Terminal bifurcation normally found in both sexes, on the fourth, fifth and sixth rays of the anal fin were partially or completely absent in treated animals. Absence of fourth ray bifurcation is the most frequent anomaly, absence of the sixth is the least frequent.

(B) *Action of testosterone propionate*

In this group gonopodia of treated animals are also shorter than the normal. The ratio total length/gonopodial length averages 4.7, that is, nearer to the same ratio in normal adult males than that of the pregneninolone treated group. Treated males show more typical gonopodia than treated females. In males the average number of ray segments is practically equal, if not superior to that found in normal males. In females, on the contrary, and in spite of the normal accomplishment of differential growth of the fin rays, the number of ray segments is much reduced. In treated males, the average number of ventral spines is greater than that of normal adult males, about 19. In treated females the average number of ventral spines is only 9. Dorsal spines and terminal hooks are absent in gonopodia of almost all treated females. The prepuce is very slightly developed, and is sometimes completely absent. In this experimental group the hormone does not seem to have any effect on terminal bifurcation of anal fin rays.

(C) *Action of transdehydroandrosterone*

In this group, although the gonopodia are slightly shorter than the normal, they are practically typical. The ratio total length/gonopodial length is equal to 4.5 and 4.2 in treated males and females respectively. Treated males have fully developed gonopodia which are identical to those of normal males in all specific characters. The average number of segments of the third ray is exactly the same as that found in normal mature males. In treated females, this hormone induced (partially) the development of the first phase of gonopodial formation, in a typical manner i.e. the phase of differential growth

of the fin rays. The average number of ray segments is less than normal and the structures are devoid of specific gonopodial characters. None of the treated females has developed a gonopodial prepuce. The hormone had no effect whatsoever, on the terminal bifurcation of the anal fin rays.

(D) *Action of methyl testosterone:*

In both sexes, the anal fin was modified in a very aberrant way, resulting in the development of an extemely atypical and short gonopodium. The ratio total length/gonopodial length averages approximately 5.5, i.e. very near to that of the pregneninolone group. In males as well as in females, the anal fin rays have undergone almost no differential ray growth. On the other hand, the average number of fin ray segments is greatly reduced. The mean number of third ray segments is about 17 in both sexes. The gonopodial prepuce is very poorly developed, sometimes not at all. While the number of ventral and dorsal spines of the third rays is extremely reduced, the terminal hook of the fifth ray is present in almost all treated individuals of either sex. The hormone (at this dose) seems to produce a strong inhibiting action on terminal ray bifurcations; individuals possessing any terminal bifurcation are very few.

Discussion

According to Weiss's theory on the antagonism between factors controlling development and those controlling differentiation, gonopodial development is accomplished in 2 phases:

a—Growth phase, followed by
b—Differentiation phase.

If a young female is treated with a low dose of androgen, we induce in such a female the beginning of the growth phase. If, on the contrary, a high dose of androgen is administered, the growth phase is more or less suppressed (according to the dose used) and the differentiation phase is induced, resulting in the development of an aberrant gonopodial structure, greatly reduced in size.

Our results show that identical doses of various androgens induce the development of gonopodial structures compared to those obtained by the administration of various doses of the same androgen.

A dose of 0.03 mg of transdehydroandrosterone (3 times a week) induces the accomplishment of the growth phase and the gonopodia obtained are completely devoid of specific gonopodial structures. The same doses of pregneninolone or methyl testosterone inhibit the differential growth of anal fin rays to a great extent and induce the development of specific gonopodial structures corresponding to the differentiation phase.

Conclusions

1. Androgenic substances exert a considerable inhibitory action on somatic growth. This action seems to be an indirect one through the hypophysis.

2. The results obtained are in perfect agreement with Weiss's well known theory on the antagonism between factors controlling growth and those controlling differentiation.

3. For the same dose of various androgens, the order of effectiveness of these substances in producing in females, a more or less typical gonopodial structure, is the following: Transdehydroandrosterone, testosterone propionate, pregneninolone and methyl testosterone. Transdehydroandrosterone induces the development of the most typical gonopodial structure and methyl testosterone induces the least typical one.

4. Identical doses of various androgens administered to females from birth, induce different degrees of gonopodial development, corresponding to different levels of response.

5. Young female anal fins seem to possess the same evolutive potentialities for gonopodial formation as those of the male.

Summary

An attempt has been made to determine the nature of the inhibiting action of androgens on somatic growth in young Lebistes reticulatus treated from birth. The experimental results seem to show that this action is an indirect one through the hypophysis. On the other hand an analytical study of differential effects of various androgens was made showing that identical doses of various androgens, administrated to females from birth, induce different degrees of gonopodial development corresponding to different levels of response.

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RETINAL TRANSPLANTATION FROM FETAL TO MATERNAL MAMMALIAN EYE[1]

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INTRODUCTION

STUDIES of the growth, differentiation and interactions of the tissues of vertebrate lateral eyes have been extensive in scope and productive of significant concepts of developmental mechanisms. Nevertheless, aside from studies of the retina in tissue culture, retinal regeneration and ocular transplantation, investigations of the developmental properties of retinal tissue are relatively meager and the approach employed in our studies apparently has not been made previously. Transplantation of relatively undifferentiated fetal retinal tissue to the maternal rat's anterior chamber has enabled us to learn more concerning retinal behavior and phenomena in the host's eye that may facilitate retinal transplantations and possibly the restoration of sight in adult mammals.

The anterior chamber is able to maintain transplanted tissues for long periods of time and has been used for this purpose in studies of many tissues other than retina. Success is dependent on the natural presence of adequate nutrients and antigenic isolation (Appel, '42; Overman and Friedewald, '50). Our aims were: (1) to determine whether a retinal transplant can be maintained and will differentiate in the anterior chamber of an adult mammal; (2) to compare the growth and differentiation of fetal retinal elements in the adult rat anterior chamber with those of the retina in its normal environment as described by Detwiler ('32) in the same strain; (3) to determine the effects of the retinal transplant on the host eye, and (4) to evaluate the interaction of transplant and host eye in terms of conditions favoring replacement of retinal tissue by transplantation.

Materials and Methods

1. *The Fetal Retina.*

Rat retina from the 16th embryonic day shows very little cytological differentiation (fig. 3) and was used as the transplanted tissue. The internal limiting membrane has not formed; and Müller's fibers have not completed their development. Instead, this zone is still occupied by the vasa hyaloidea propria. The nerve fiber layer is partially developed, particularly in the fundus; and here processes are arriving at the optic stalk. The ganglion cells are identifiable only in this region. On the whole, the retina consists of a single, broad zone of nuclei similar to the outer neuroblast layer of a 15 mm. human embryo. Mitotic cells occur along the external edge.

2. *Animal and Surgical Procedures.*

Fifty female Long-Evans rats, 90–180 days old, were transplant recipients (45) and controls (5). They were individually caged in a room receiving 12–14 hours of artificial light in a daily photoperiod. Four male Long-Evans rats, used for breeding, were placed singly overnight with the females which had attained a proestrous condition following two normal cycles, as determined by vaginal smears (Long and Evans, '22).

Seventeen days after mating, surgery was performed with ether anesthesia. The left horn of the uterus was exposed via an incision one and a half centimeters long in the clipped and alcohol-rinsed left side of the abdomen. The left uterus was drawn through the opening, and was kept moist in physiological sodium chloride solution. Ligatures were passed just below the ostium and also near the junction of the cornu, including the left uterine artery. The left horn and its embryos were cut free with minimal bleeding and transferred to warm Ringer's solution where they remained while the abdominal incision was being closed. The left eyeball of the maternal rat was then grasped carefully and the cornea was pierced at a point next to the limbus. The escape of some aqueous automatically made room for a transplant. A single fetus was exposed in the excised uterine horn and transferred to an adjacent Petri dish containing warm saline, leaving the umbilical cord intact. The left fetal eyeball was removed to saline in a depression slide, where it was bisected equatorially by a single

cut. The posterior moiety was then halved, and one piece was trimmed free of as much sclera as possible without disturbing the retina. The trimmed prospective transplant was placed on the cornea of the maternal rat's left eye adjacent to the incision made previously and tucked into the anterior chamber. The entire orbit was then bathed with saline and dried with a cotton swab, and the surface of the cornea was coated with a three per cent nitrocellulose solution with a camel's hair brush to seal the incision. Ophthalmic examinations were performed periodically with the aid of ether anesthesia. Initial clouding of the aqueous, due to the introduction of blood at transplantation, rapidly disappeared. However, examination of areas beyond the pupil was soon handicapped by growth and blocking by the transplant. In all cases the host animals carried their embryos to term through a gestation period of normal length (21 days) and cared for their young satisfactorily.

The eyeballs containing transplants were removed at post-operative days 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, and 50. The older transplants (32–50 days) included 4 of each age, the 20-day ones 3, and the younger transplants (5–18 days) 2. Two of the three eyes taken after twenty days differed from the others in that one contained embryonic cerebral and the other cerebellar tissue in place of retina. In addition, the eyeballs of three sham-operated females were removed 10, 20 and 30 days after surgery.

3. *Histological Procedures.*

Eyes were removed and immediately placed in Kolmer's fixative (Lillie, '54) for about five minutes. Two small incisions were made, one in the cornea and one through the sclera, choroid and retina, so that more rapid passage of fixative and subsequent solutions would be possible. The transplant and the lens were untouched during this procedure. Further fixation for 24 hours in Kolmer's solution was followed by washing in tap water, dehydration through ethanol solutions, clearing in cedarwood oil and benzene, and embedding in paraffin in vacuo. All eyes were serially sectioned at 7 or 10 microns, either perpendicular or parallel to the equatorial plane. Due to our primary interest in the retinal nerve fibers, Davenport's modification (1939) of Bodian's protargol method was used, with the additional modifications of decreased time in silver nitrate and protargol and increased washing times in double-distilled water.

Results

1. *Compatibility, Growth and Differentiation of the Transplant.*

Forty, or 89%, of the 45 animals that received retinal transplants maintained the grafted tissue either wholly or in part. Of the 5 transplants that did not survive, 2 were found at 30 days and 3 at 35 days after operation. The appearance and increase of the grafted tissue, prior to degeneration, suggests, however, that these had been maintained postoperatively for about 25 days.

Since all retinal transplants were made on the 16th day of fetal life and birth occurs usually on the 21st day, transplant ages as discussed below are equivalent approximately to postnatal ages plus 5 days.

Days 5 and 6. The transplant at this stage looks nearly the same as when it was taken from the fetus. There is a slight separation of the ganglion cell layer from the outer cellular layers by the inner plexiform layer, but there is still no distinct separation of the rod and bipolar cell layers. Although the stratum opticum is evident, the orientation of the short fibers composing this layer is not readily apparent at this time. Numerous retinal mitoses in the primitive nuclear zone preclude accurate nuclear measurements. The thickness of the cell layers and the nuclear diameters are given for this and subsequent stages in Table 1.

Day 7. At this stage photoreceptor processes (presumably rods) are first seen and extend 0.4 microns beyond the zone of mitotic nuclei. Increases in the thickness of the ganglion cell layer and nuclear diameter also occur (Table 1). In the normal rat retina at this stage, rod processes are not yet clearly differentiated, but the external limiting membrane is more evident.

Day 8. This is the only time at which all of the retinal layers can be seen and measured in the transplant. In the transplant, rod processes are little more than 1.0 microns long as compared with 1.5 microns in the intact normal retina (Detwiler, '32) of this age. Melanin pigment is present in the transplant's pigment epithelium at this stage.

Day 9. The emigration of the ganglion cells, commencing at this time, prohibits the taking of consistent measurements of the thickness of this layer. Fibrous connections between the strata are maintained,

however. Rod, external nuclear, and internal nuclear layers are all increased in thickness.

Day 10. The differential staining of the nuclear layers of the transplanted retina aids in their recognition at this stage and resembles that of the maternal host's retina.

Day 12. The nuclei of the bipolar cells reach their maximum size, while the mean nuclear diameter of the ganglion cells continues to increase for another eight days (Table 1).

TABLE I
MEASUREMENTS OF TRANSPLANT ELEMENTS IN MICRA

Transplant age at recovery (days)	Thickness of Retinal Layers				Nuclear Diameters		
	Rods	External Nuclear	Internal Nuclear	Ganglion	Rod	Bipolar	Ganglion
5	—	—	—	14.3	—	—	4.6
6	—	—	—	14.4	—	—	4.6
7	0.4	—	—	15.1	—	—	4.8
8	1.0	18.1	12.3	15.6	4.0	5.0	5.0
9	1.3	19.2	12.8	—	4.0	5.0	5.3
10	1.9	21.1	13.9	—	4.1	5.2	5.6
12	2.0	24.0	14.3	—	4.0	6.5	5.9
14	5.3	25.2	15.1	—	4.1	6.5	6.1
16	8.7	28.7	20.4	—	4.2	6.6	6.8
18	15.4	32.3	23.9	—	4.2	6.5	7.2
20	19.3	34.0	—	—	4.2	6.4	7.8
25	—	—	—	—	4.2	6.5	6.7
30	—	—	—	—	4.1	6.6	5.4
35	—	—	—	—	4.1	6.6	5.0
40	—	—	—	—	4.0	6.5	5.1
45	—	—	—	—	4.0	6.6	5.0
50	—	—	—	—	4.1	6.5	5.3

(—) Indicates that either the element had not differentiated as yet or that its migration rendered measurements not feasible.

Day 14. At about this time, or a day or two later, a period of accelerated rod growth begins in the transplant as well as in the intact developing eye (fig. 1).

Day 18. The accelerated growth phase of the rods in the transplant continues, but from this time on, the length of the rod processes in the transplant falls behind that of the intact developing eye (figs. 4, 5). The bipolar cells begin their movement towards the periphery of the transplant, thus preventing the subsequent measurement of the inner nuclear layer.

Day 20. This is the last time at which the rod nuclear layer main-

tains its continuity. And at this time the rod processes are 19.3 microns long as compared with a normal of 23.5 microns. The greatest mean value for ganglion cell nuclear diameter occurs at this time; there is subsequent decrease for about 10 days (Table 1).

Days 25 to 50. The transplant's retinal cells emigrate posteriorly during this period. The layers lose their integrity and rod cells can

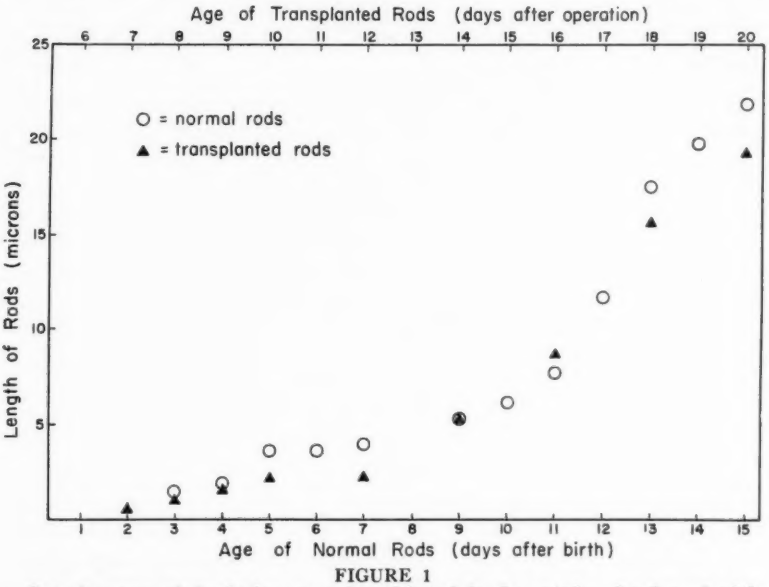

Age of Transplanted Rods (days after operation)
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
25
20
15
10
5
○ = normal rods
▲ = transplanted rods
Length of Rods (microns)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Age of Normal Rods (days after birth)

FIGURE 1

Growth curves of developing rat rods, cultivated in the anterior chamber of adult hosts' eyes and in intact postnatal eyes (data for the latter from Detwiler, '32).

not be easily identified. The mean nuclear diameter of the ganglion cells drops markedly, probably due to the differential survival of the smaller, more diffuse cells of this type.

2. *Movements and Fates of Retinal Cells.*

Day 5. Even at this stage it is found that the major part of the transplant has moved through the pupil to the posterior surface of the host's iris. The route through the anterior chamber is marked by vascular tissue, erythrocytes and lymphocytes. Along the margins of

this vascular proliferation there is fibrous tissue extending from the point of corneal penetration, through the pupil, and to the transplant. The transplant is firmly attached to the host's iris by extensions into the latter's stroma.

The internal organization of the transplant at this time consists of, from iris to vitreous: (1) discontinuous pigment epithelium, (2) inner nuclear layer, (3) inner plexiform layer, and (4) ganglion cell layer. The outer surface of the transplant, especially posteriorly, is covered with fibroblasts, with considerable mitotic activity and distinctly different cytologically from the ganglion cells. The fibroblasts, with an irregular nuclear membrane and multiple nucleoli, have apparently given rise to phagocytes. And from the zone of fibroblasts and short axons of ganglion cells, fibers of uncertain origin extend across the posterior chamber to the lens capsule.

Days 6 and 7. Penetration of the lens by cells from the transplant occurs now as well as in all later stages. Fibers and fibroblasts are seen under the lens capsule for a few microns on either side of the point of entrance. These elements also extend through the zonular region following the outside contour of the lens.

Day 8. The transplant's fibrous processes have by this time extended to an area of the lens opposite the ora serrata outside of the capsule; and more fibers and fibroblasts have contributed to the internal contents of the host's lens and form layers of fibers here against the internal surface of the lens capsule.

Days 9 and 10. The fibrous processes of the transplant extend to and along the anterior limiting layer of the vitreous and form a major accumulation of fibers on the posterior surface of the host's lens. Along with this growth, fibers can be traced to the host's retina, to the latter's internal limiting membrane and nerve fiber layer where fibrous endings appear to lie primarily on channels of the capillary bed. Some isolated ganglion cells are seen a short distance from the main mass of the tranplant and within the fibrous material extending posteriorly from the transplant.

Days 12 to 14. The cells of the transplant form rounded, rosette-like masses with rods innermost and a loss of the pigment epithelium from this area. The pigment epithelium appears to be contributing rather to the stratified squamous epithelium adjacent to the transplant. This epithelium forms variably a capsule in scattered spots

from anterior to posterior chambers. The formation of rosette-like ovals of retina has no obvious ill effects on the transplant, since mitoses continue and nuclei appear to be normal. By this time many ganglion cells and fibroblasts occur in the fibrous extensions from the transplant.

Days 16 and 18. Aggregations of ganglion cells and fibroblasts lie on the posterior surface of the lens and send processes into the vitreous especially via the canal of Cloquet.

Day 20. Dissolution of the organized inner nuclear layer of the transplant occurs and the affected cells appear to follow the path around the lens taken by the ganglion cells. The processes of the ganglion cells behind the lens are longer.

Day 25. The processes of the ganglion cells, behind the lens coursing along the hyaloid canal or the canal of Cloquet have joined an induced structure extending from the host's fundus, a reconstituted hyaloid artery (fig. 2). This vessel or vessel system is first observed on day 5 (fig. 8) and is found in all older eyes bearing a retinal transplant. The transplant's rod cell layer is disorganized by this time and rod and bipolar cell nuclei are scattered along the emigration route.

Day 30. Only 2 of the 4 30-day old transplants appear to be healthy. In these the rod and bipolar cells are still emigrating and are concentrated in the equatorial region of the lens. Behind the lens the connection between the ganglion cells' axons and the reconstituted hyaloid artery is increasing in width and increasing in its content of ganglion cell bodies.

Day 35. In the one surviving transplant of the four fixed at this stage, the inner and outer nuclear layers have reached the posterior side of the lens, in a superficially haphazard arrangement of rod, bipolar and ganglion cells. The majority of the ganglion cells, however, now lie in the connective tissue around the reconstituted hyaloid artery and associated vessels, particularly in the vicinity of the attachment to the host's retina. The outer covering of the reconstituted hyaloid vessels and attached cells is formed by fibroblasts, many in mitosis. Between this investment and the vessels, fibers extend from healthy ganglion cells toward the host's optic disc. It is concluded that these are diffuse ganglion cells, primarily because of their nuclear size and structure and the diameter of the perikaryon (7.3–8.2 microns).

Day 40. The ganglion cells of the transplant are now concentrated even closer to the host's retina and darkly stained fibers pass among them and form glomeruli on their cell bodies.

Days 45 and 50. The ganglion cells are concentrated around the basal attachment of the reconstituted hyaloid vessels and fibers pass

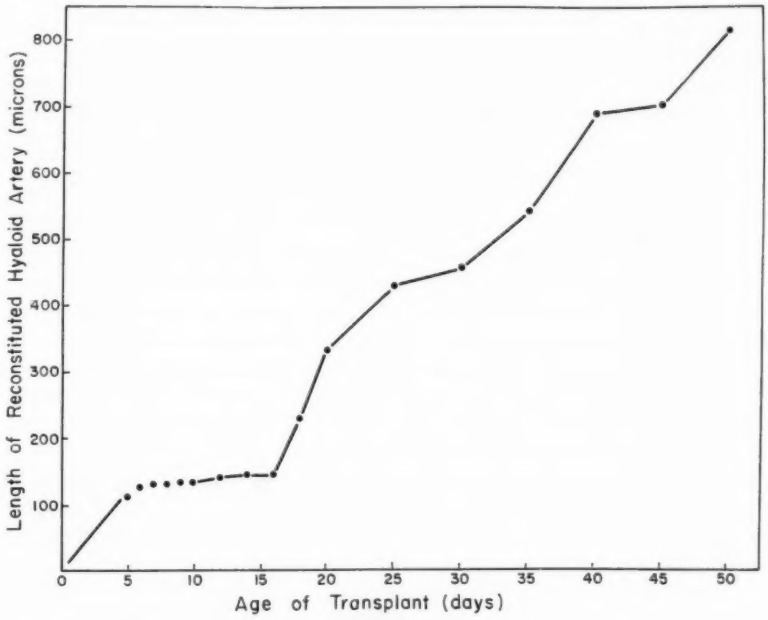


FIGURE 2

The growth in length of the reconstituted hyaloid vascular and connective tissue structure in the hosts' eyes according to time after transplantation of fetal retina to the anterior chamber.

from their vicinity into the host's optic nerve through the lamina cribrosa. Cells from the transplant's inner and outer nuclear layers are concentrated just behind the lens, but extend posteriorly also about halfway toward the host's retina in the tissue around the hyaloid vessels. Astrocyte-like neuroglial cells of uncertain origin and their processes occur among the transplant's ganglion cells close to the host's optic nerve head. Fibroblasts have formed a connective tissue

sheath around most of the length of the pillar of tissue formed by the hyaloid vessels and the surrounding cells. Emanating through this connective tissue sheath fine fibers of uncertain nature pass to other points on the host's retina and papillary extensions from the latter mark the attachments.

3. *Effects Induced in the Host's Eye.*

Iris and cornea. Five or six days postoperatively the cornea's nitrocellulose coating, applied after surgery, is seen to have been shed and the region of corneal puncture contains hypertrophied epithelial cells surrounding damaged tissue. The outer cellular layers in this area are squamous and without visible nuclei. Peripheral to the hypertrophied epithelial cells is an area of increased vascularity, extending from conjunctival blood vessels. Bowman's membrane has not yet regenerated, but the substantia propria is knitting inward. In this, as well as in all subsequent stages, Descemet's membrane does not regenerate across the area of corneal puncture. The area of the iris directly below the wound contributes fibroblasts which aid in the formation of the plug in the corneal substantia propria. Between seven and nine days after operation, blood vessels extend from the iris to this penetration site. Vessels coursing through the anterior chamber of the maternal eye along the fibroblast meshwork were identified as arising in the iris and have a characteristic structure in which the adventitia is separated from the endothelium by a distinct space. During this period, these vascular channels penetrate also the core of the retinal transplant on the posterior border of the iris.

Between ten and sixteen days after operation the corneal epithelium completely heals and its hypertrophy disappears. Bowman's membrane regenerates and the substantia propria loses its vascularity. The corneal endothelium has not yet become continuous across the lesion and a fibrous connection between iris and corneal substantia propria still exists. Although no blood vessels are seen in this tissue strand beyond twenty days, connective tissue fibers continue to be present even fifty days after operation.

Sporadic in occurrence throughout the series are balls of stratified squamous epithelium with hollow cores and luminal sloughing of cells. It seems probable that these are derived from fragments of corneal epithelium that were accidentally transferred and trapped

in the anterior chamber during paracentesis. When these epithelial balls occur, tissue connections between them and the corneal wound are found by tracing relations though the serial sections.

Lens. In the earliest eyes bearing retinal transplants the lens capsule at a point opposite the transplant has an uneven edge and is penetrated by minute fibrous processes from the transplant. In sham-operated eyes the lens capsule remains smooth and unpenetrated, while in the twenty-day old cerebral transplant, fibroblastic penetration of the lens similar to that of the retinal transplants occurs. In all eyes bearing retinal transplants six days or older, fibroblasts emanating from the transplant penetrate the lens and exhibit a maximal proliferation about twenty days after operation. The interior of the lens appears sclerotic beyond this time.

From the twenty-fifty to the fiftieth day, the lens is displaced anteriorly in eyes bearing retinal transplants but not in sham-operated eyes. By the fiftieth day the anterior chamber is nearly obliterated by this movement.

Retina. From five to eighteen days after retinal transplantation the strata of the maternal host's retina appear to be normal. On the twentieth day, however, some cells appear to be degenerating in various places throughout the photoreceptor layer, and especially near the optic nerve. Concurrently, nuclear shrinkage and disorganization of cell elements occurs at scattered points in the outer nuclear layer of the host's retina. The other strata remain normal in appearance throughout the experiment. Though rods continue to be present in the host's retina through the fiftieth day, at no time was their complete organizational integrity regained.

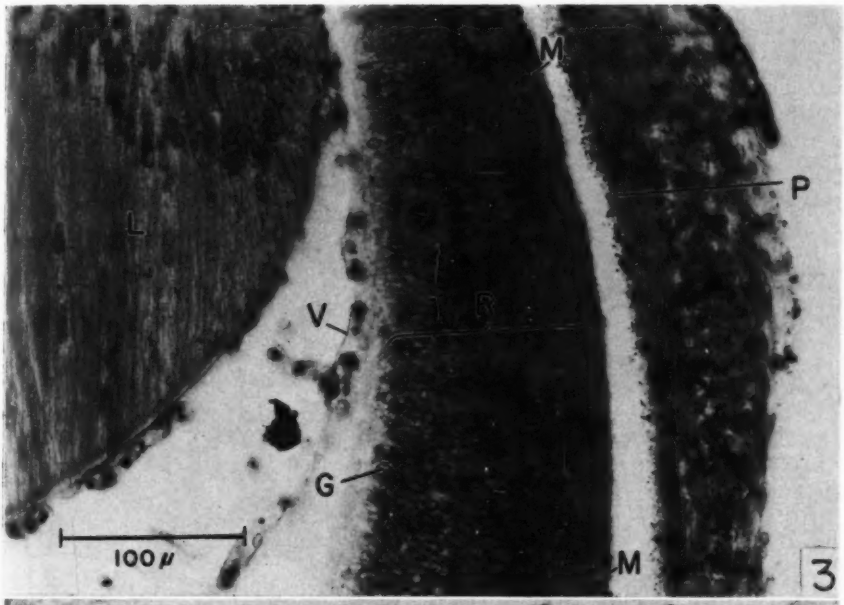
In eyes bearing retinal transplants as young as five days a small vascular extension enters the vitreous humor from the central artery of the host's optic disc (fig. 8). The growth of this vascular structure continues through the fiftieth day (fig. 9) and is graphed in figure 2. By the twentieth day it is evident that this vessel is following the hyaloid canal or canal of Cloquet through the vitreous and is a reconstituted anatomical equivalent of the fetal hyaloid artery. At this time also the anterior end of this canal is occupied by cells migrating from the retinal transplant as described above. At twenty days the growing, reconstituted hyaloid artery is about 330 microns long and is covered with a layer of fibroblasts and associated con-

PLATE 1

Explanation of Figures

3. The 16-day-old fetal eye. R = retina and the layer forming the transplanted tissue; G = ganglion cells, beginning to be visible, but their axons have not formed a fibrous layer as yet; L = fetal lens; M = mitotic cells in the proliferative or future rod layer of the retina; P = pigment epithelium, with sparse melanin granules at this age; V = vasa hyaloidea propria.

4. The fetal retinal transplant and adnexa of the host's eye in frontal section 18 days after the operation. C = iris capillaries; F = fibroblasts investing the migrating ganglion cells and invading the host's lens; G = ganglion cells migrating posteriorly from the transplant; I = anterior surface of the iris; IN = inner nuclear layer of the transplant; LC = penetrated lens capsule; ON = outer nuclear layer of the transplant; P = photoreceptor layer of the transplant (enlarged in next figure).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3

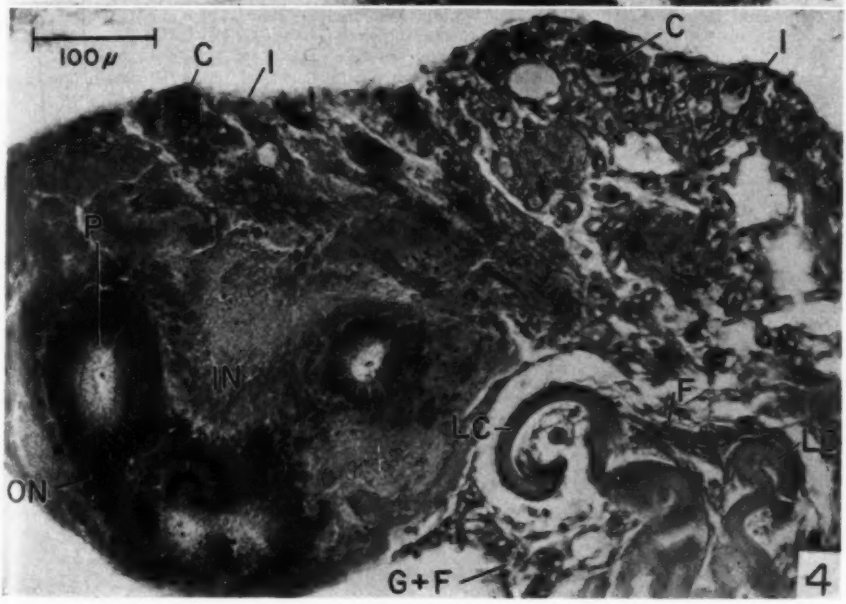
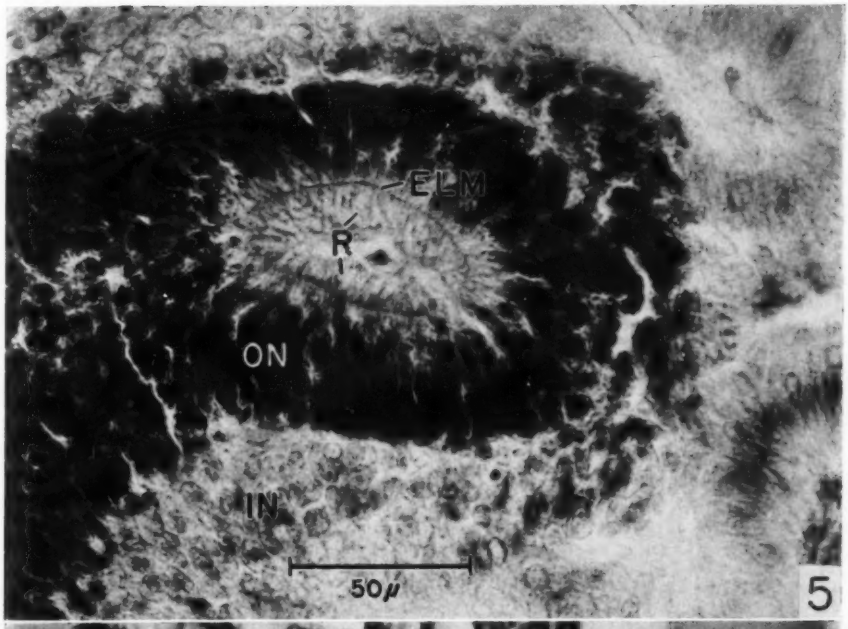

100 μ
C
I
C
I
P
IN
F
LC
L
ON
G+F
4

PLATE 2

Explanation of Figures

5. Outer nuclear layer and rods of the 18-day-old fetal retinal transplant (enlarged from previous figure). ELM = external limiting membrane; IN = inner nuclear layer; ON = outer nuclear layer; R = rod processes projecting into a central lumen.

6. The migrating ganglion cells of a 40-day-old transplant. BF = process from migrating bipolar cells; G = ganglion cells; LC = disrupted lens capsule; PG = ganglion cell with pyknotic nucleus.


ELM
R
ON
IN
50μ
5

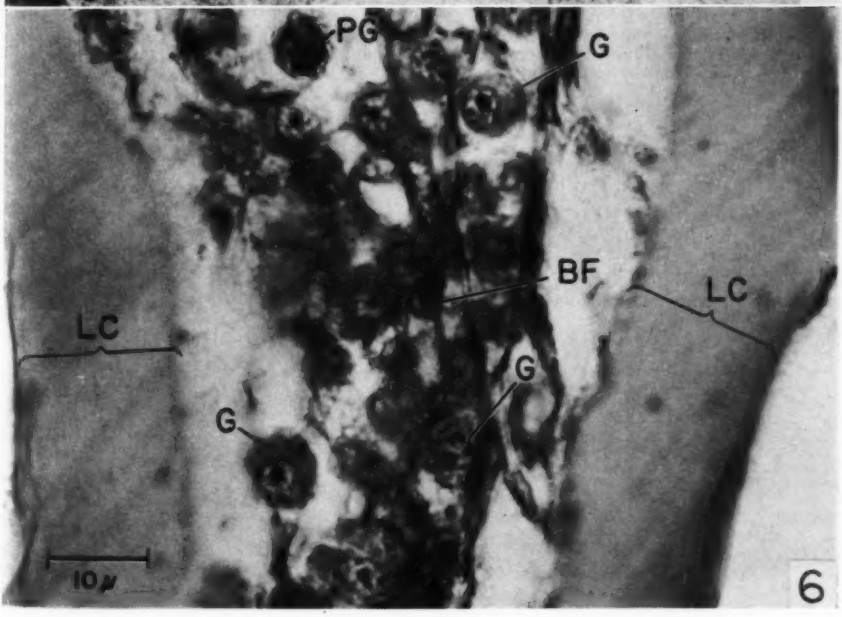
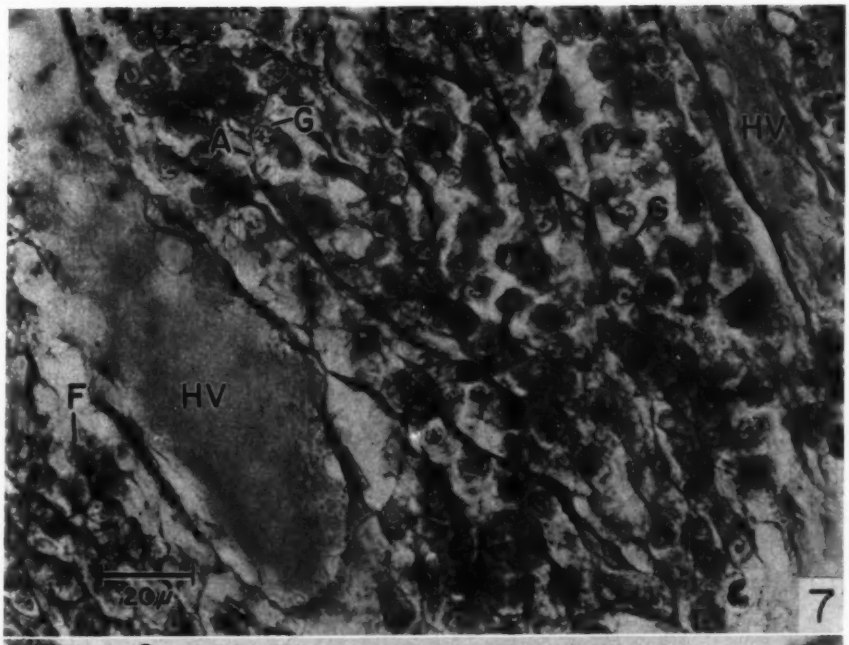

PG
G
BF
LC
LC
G
G
10μ
6

PLATE 3

EXPLANATION OF FIGURES

7. The ganglion cells at the base of the reconstituted hyaloid vessels in a 50-day-old transplant. A = axon of ganglion cell coursing toward optic nerve head (beyond lower right); F = fibroblast; G = ganglion cells from transplant; HV = hyaloid blood vessels; many of the deeply stained fibers among the ganglion cells are from bipolar and astrocyte-like glial cells.

8. The retina, optic nerve head and reforming hyaloid vascular bud of the host's eye 5 days after retinal transplantation. A = artifact splitting of retinal layers; CA = central artery; G = ganglion cell layer of host's retina; HB = hyaloid vascular bud; IN = inner nuclear layer; NF = nerve fiber or ganglionic axon layer; O = optic nerve head; ON = outer nuclear layer; P = photoreceptor layer.


G
A
HV
F
HV
20μ
7

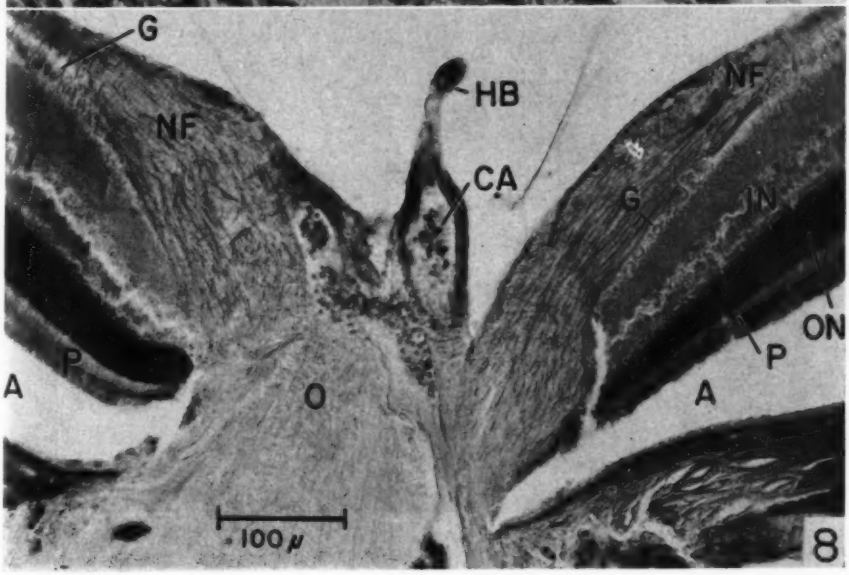
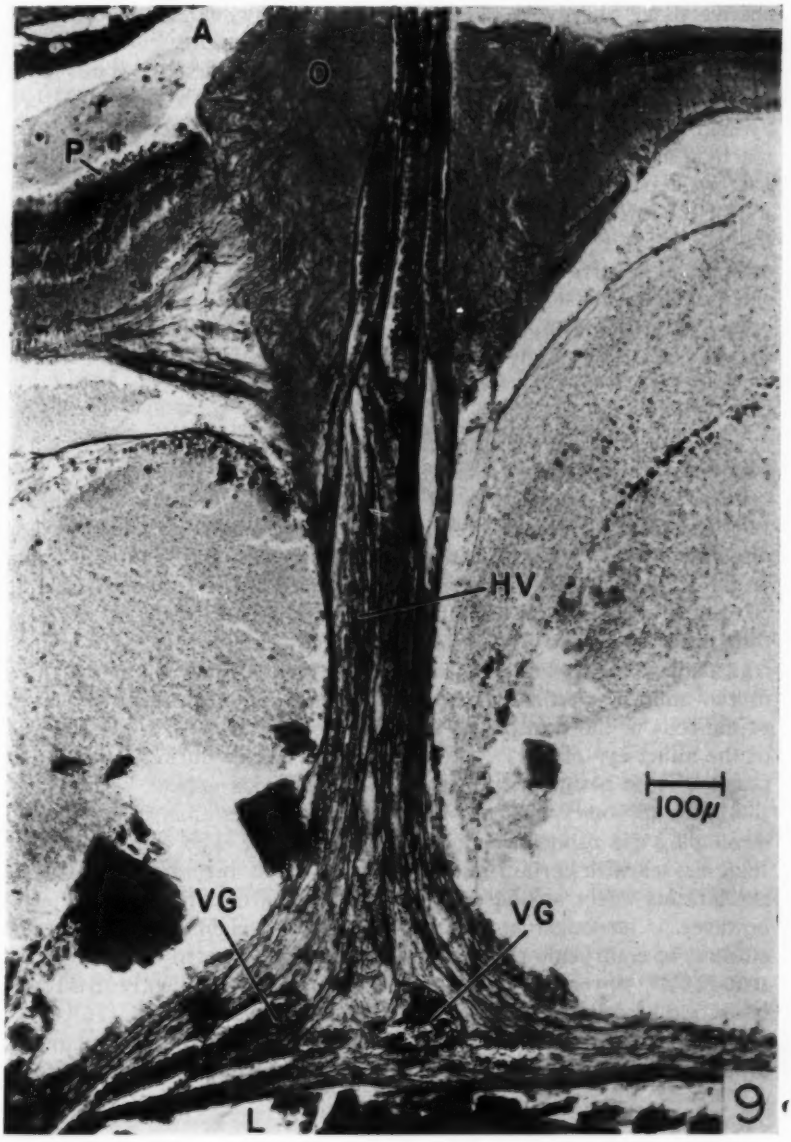

G
NF
HB
CA
NF
G
IN
ON
P
P
A
A
O
100μ
8

PLATE 4

Explanation of Figure

9. The reconstituted hyaloid vascular system and adnexa at 50 days. A = artifact splitting; HV = hyaloid blood vessels; L = lens; O = optic nerve head; P = photoreceptor layer; VG = vascular growth around the back of the lens from the hyaloid system.


A
O
P
HV
100μ
VG
VG
L
9

nective tissue fibers. At twenty-five days the hyaloid vessel contacts processes of cells from the transplant. Between twenty-five and fifty days transplant and maternal hyaloid elements mix, as transplant ganglion cells progress posteriorly to the maternal retina and the hyaloid tissue and artery extend to the posterior surface of the lens reaching a length of 818 microns and a width of 128 microns at the narrowest points (fig. 9).

It is of interest that in those eyes in which the retinal transplant did not survive (i.e. 30 and 35 days), reformation of the hyaloid artery had occurred but either did not progress beyond, or had subsided to, the level seen at twenty-five days. In neither sham-operated eyes (30 days) nor eyes containing cerebellar or cerebral transplants (20 days) are there any reforming hyaloid vessels.

Discussion

The differentiated transplanted retina shows in the following characteristics a remarkably close structural similarity to the adult rat retina from the fundic area to at least midway to the ora serrata: (1) The mean depths of the inner and outer nuclear layers prior to dissolution and migration are respectively 23.9 and 34.0 microns. In the adult rat retina Detwiler ('32) found that these layers measure 31 and 40 microns in the fundus, while closer to the ora serrata, they measure 18 and 31 microns. (2) The ganglion cell layer in the transplant contains several strata of these cells, which is not the case in the anterior portion of the adult retina. (3) The growth curve of the rods in the transplants is similar to that of rods in the fundus of the intact eye (fig. 1). There is some precocity during the earliest phase of transplant differentiation since the rods appear earlier and the inner and outer nuclear layers separate earlier than in the intact developing eye as described by Detwiler ('32). The contact of the fetal tissues with certain adult ocular materials may have had some accelerating effect on the early phases of differentiation. This effect, however, is no longer apparent after seven or eight days. In tissue cultures of embryonic vertebrate eyes it has been noted that there are remarkable powers of self differentiation, but frequently with an inhibition of cellular multiplication (Strangeways and Fell, '26).

The most critical period for the maintenance of the transplant is around twenty days, when structural reorientation and migration of

the cellular elements in the transplant begins. During the period from twenty-five to fifty days there appears to be a selective survival and migration of the small, diffuse type of ganglion cell. At the end of this period at least some of these ganglion cells send their processes into the host's optic nerve, as in the normal retina. Processes ending in glomeruli on the cell bodies of the posteriorly moving ganglion cells support the belief that these cells from the transplant are still in contact with bipolar cells found at more anterior points along the emigration route. In tissue cultures, such a relatively fixed polarity has been claimed for the ganglion cells in retinal explants that the course of the ganglion cells' axons could be predicted (Vinnikov, '46). Nevertheless, mechanical factors frequently appear to be dominant influences on the courses taken by nerve fibers in tissue cultures manipulated to test chemical and electrical influences as well (Weiss, '34). And the primary path followed by the axons of the ganglion cells in our study might well be interpreted in this way.

The normal presence of cones in the rat's retina has been noted by Walls ('34). While we were unable to find cones in the transplanted retina, it is likely that the normal preponderance of rods in this noctural species and the nature of our staining technique are primarily responsible for this impression.

The formation of rosettes by the photoreceptor layer in the rat retina has been noted previously in tissue cultures both of embryonic retina and older whole embryonic eyes as well as in trephined eyes of 5-day old animals (Tansley, '33). It appears likely that disturbance or reduction of intraocular pressure as well as intrinsic properties of the retina are responsible for this process.

The invasion of the host's lens by retinal transplants, particularly fibroblasts, does not appear to be specific since the cerebral transplant had the same activity. This effect on the lens does not seem to be due to abrasion at operation, since it is not apparent until after the early stages of explant proliferation and it is absent altogether in sham-operated eyes.

Our results support the belief that the reconstitution of a hyaloid artery is a specific effect of retinal tissue placed in the anterior portion of the eye. The transplant's forsaking of a lavish vascular supply from the iris for a distant developing supply from the retina is no less dramatic than the reformation of this latter vascular system.

Nevertheless, the anatomical path of this new blood supply from the host's retina may possibly be dictated by the peripheral, presumably firm, attachment of the vitreous body to the ciliary epithelium (Wolff, '51). The effects of peripheral detachment or partial removal of the vitreous on the anatomical relations of transplant emigration and blood supply from the host's retina would be interesting to determine in this connection. The orientation of the retinal transplant in our series parallels the normal developing retina if the vascular relations are noted. The vascular system from the iris is analogous to a choroid supply, and the reconstituted hyaloid system an extension of the central artery and its retinal branches. During the first fifteen days the reforming hyaloid system grew on the average of 10 microns per day. Between sixteen and eighteen days a distinct acceleration in the growth of the hyaloid system begins, amounting to 19.1 microns per day (Fig. 2). This rate is not excessive when contrasted with that of fetal retinal vessels, ten microns per hour (Michaelson, '54). In our studies accelerated hyaloid growth appears to coincide with the arrival of ganglion cells at the posterior surface of the lens.

The feasibility of retinal repair in adult or old mammals by means of fetal retinal grafts seems enhanced by the results which we have described. Further experiments in which the vitreous is manipulated and selected retinal quadrants are removed before grafting seem promising on this basis. Also, experimental techniques to determine the path taken centrally by transplanted ganglion cells' axons now seem to be in order.

Acknowledgments

We are grateful to Drs. W. E. Berg, R. M. Eakin and G. L. Walls for their reading of the manuscript and helpful comments concerning it, and to Mr. Victor Duran and Mrs. Emily Reid for the photomicrography and final rendition of the figures.

Summary

Pregnant female Long-Evans rats received retinal transplants in the anterior chamber of one eye from one of their 16-day-old fetuses. Microscopic studies of serial sections of the hosts' eyes and their retinal implants fixed from five to fifty days after surgery demonstrate the following:

(1) The retinal transplant survived in 89 per cent (40/45) of the eyes.

(2) The growth and differentiation of the transplant leads to a mutually normal arrangement of its retinal layers and a nearly normal thickness of these layers by the twentieth day after transplantation.

(3) The photoreceptor layer of the differentiated transplant consists, at least largely, of rods which form rosettes, but still exhibits a growth rate similar to that of rods in the intact eyes of postnatal rats of equivalent ages.

(4) Five days after operation most of the transplanted tissue has moved through the pupil and is attached to the posterior surface of the host's iris. Starting about nine days after operation a cellular emigration from the transplant, around the lens, through the hyaloid canal, and toward the optic nerve occurs.

(5) The posteriorward cellular movement from the transplant is led by smaller, diffuse ganglion cells and fibroblasts. The former *send at least many* of their axons into the host's optic nerve 45 to 50 days after operation.

(6) A progressive reconstitution of the host's hyaloid artery occurs from before 5 days until at least 50 days after the operation and is a specific effect of the retinal transplant.

(7) A destructive fibroblastic invasion of the host's lens from the transplant appears to be a nonspecific effect of the retinal tissue.

(8) Starting about 20 days after operation degenerative changes occur in the host's photoreceptor layer at scattered points, especially near the optic disc.

(9) While we were not successful in integrating transplanted and host retinal elements into a normal retinal structure, our results do show the feasibility of fetal retinal transplantation as an instructive procedure that may provide basic information as well as lead to techniques for retinal repair in adult mammals.

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EARLY POSTNATAL CHANGES IN NEURON PACKING DENSITY AND VOLUMETRIC RELATIONSHIPS IN THE CEREBRAL CORTEX OF THE WHITE RAT[1]

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EFFORTS to establish a sound basis for studies of relationships between structure and function in the cerebral cortex are reflected in the quantitative histological investigations of a number of authors during the past four decades or more (1-18). While many of the earlier studies, particularly those of Bok (3, 4), Haug (11, 12), Sholl (14, 15), and von Bonin (8), were extremely comprehensive and carried out in great detail, few efforts have been made to utilize their findings in experimental studies. This is probably due to the tremendous numbers of cell counts and measurements which would have to be carried out in order to determine effects of various experimental procedures on packing densities, volumetric relationships or patterns of dendritic branching as reported by these authors. The difficulties in this regard would be compounded many times in any effort to carry out developmental studies with such techniques.

Accordingly, our own recent studies (16, 17, 18) have been directed toward providing data somewhat more limited in scope, but more readily applicable as a frame of reference for experimental developmental studies in neurochemistry and radiobiology. This paper represents a continuation of our initial investigations of postnatal changes in neuron packing density and volume relationships of the cerebral cortex.

Materials and Methods. This investigation was carried out on 22 white rats of the Sprague-Dawley strain. The animals were grouped according to age at 1, 5, 10, and 20 days. The cortical area investigated

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was the cranio-caudal middle portion of area 2 (19). The brains were fixed and stained as in our previous studies (16, 17).

The nuclear and cell volumes were determined by measurement of the greater and lesser diameters of nuclei and cell bodies at ten progressively deeper and equidistant levels between the outer border of layer II and the inner border of layer VI. An ocular micrometer calibrated with a stage micrometer was used to determine the various diameters, while a point placed on the ocular micrometer was used to randomly select the cell to be measured. The cell which fell closest to the point at each successively deeper level was the one measured. Three hundred cells and their nuclei were measured in this manner at each developmental stage. The formula $\pi/6$ ABC, as described by Micklewright, *et al.* (20) was applied to the values obtained. The perikaryon volume was taken to be the mean of the differences between the cell and nuclear volumes of each cell measured.

The cell packing densities were determined by the Chalkley circle method (21) as adapted to counts of cells of cerebral cortex in earlier investigations (16, 17). This involves counting cells at ten equidistant and progressively deeper levels between the outer border of layer II and the inner (deeper) border of layer VI. The cells are counted by focusing a circle on an eye-piece graticule through a *measured depth* of tissue and counting the nuclei enclosed within the "counting chamber" thus delimited. The measured depth of tissue lies in the center of the section so as to avoid the cut surfaces of the section. Nuclei bisected by all of the borders of the "counting chamber" are evaluated as follows. Each nucleus of which more than half is included in the circle is counted as a whole nucleus. Each nucleus of which less than half is included in the circle is not counted. All nuclei of which exactly half is included in the circle are recorded and one half the number of such equally bisected nuclei is taken. While the evaluation of nuclei bisected by the walls of the counting chamber or edge of the circle is thus quite simple, the evaluation of cells bisected by the upper and lower borders or "roof" and "floor" of the counting chamber requires repeated careful focusing to determine what proportion of the nucleus is included in the chamber and hence whether or not it should be counted.

It is important in this latter evaluation to provide enough space between the upper and lower borders of the counting chamber and

the cut surfaces of the section to accommodate at least the radius of the lesser diameter of the largest nucleus in the section.

The thickness of section, diameter of circle and depth of focus employed at each developmental stage are shown in Table I.

TABLE I

Age in Days	Diameter of Circle or "Counting Chamber"	Depth of Focus (Depth of "Counting Chamber")	Section Thickness
1	20 μ	15 μ	25 μ
5	30 μ	15 μ	25 μ
10	40 μ	20 μ	35 μ
20	40 μ	20 μ	35 μ

Great accuracy in measuring increments of cortical depths for successive samples was attained through the use of a nuclear tracking mechanical stage on a Leitz ortholux microscope.

The interval between sections counted was 3 in the 1 and 5 day animals, and 5 in the 10 and 20 day groups.

TABLE II

Age (days)	1	5	10	20
No. of Animals	4	6	6	6
Mean Nuc. Vol. (μ^3)	152.58 ± 8.75	333.82 ± 19.26	420.90 ± 10.94	442.75 ± 22.49
Mean Cyt. Vol. (μ^3)	72.07 ± 3.54	183.80 ± 23.82	444.09 ± 21.96	588.56 ± 24.74
Mean Cell Vol. (μ^3)	224.65 ± 7.61	517.62 ± 42.07	864.99 ± 26.43	1031.31 ± 40.27
Mean Nuc/Cyt Ratio	2.12 ± 0.21	1.81 ± 0.17	0.95 ± 0.05	0.75 ± 0.04
Mean Gray/Cell Coef.	6.54 ± 0.35	6.97 ± 0.26	8.00 ± 0.38	8.56 ± 0.49
Mean Neuron Packing Density ($\div 10^5/mm^3$)	6.80 ± 0.321	2.77 ± 0.121	1.45 ± 0.133	1.13 ± 0.040
Mean Brain Weight (Grams)	0.33 ± 0.062	0.55 ± 0.058	0.664 ± 0.041	1.258 ± 0.012

Results. An outline of our observations is recorded in Table II, and Figs. 1 to 5. Nuclear volume (Fig. 1) increased from a mean value of 152 μ^3 in 1 day animals to 333 μ^3 at 5 days. An increase was again noted in 10 day animals with a mean value of 420 μ^3 after which no significant increase was observed, the final value in the 20 day group being 442 μ^3.

Mean perikaryon volume (Fig. 1) increased from 72 μ^3 at one day to 183 μ^3 at 5 days, then increased more rapidly to 444 μ^3 in the 10

day group, after which the final increase to 588 μ^3 in the 20 day group was observed.

The mean cell volume (nucleus + perikaryon) (Fig. 1) increased from about 224 μ^3 at one day to 517 μ^3 at 5 days, 864 μ^3 at 10 days and 1031 μ^3 in the 20 day group.

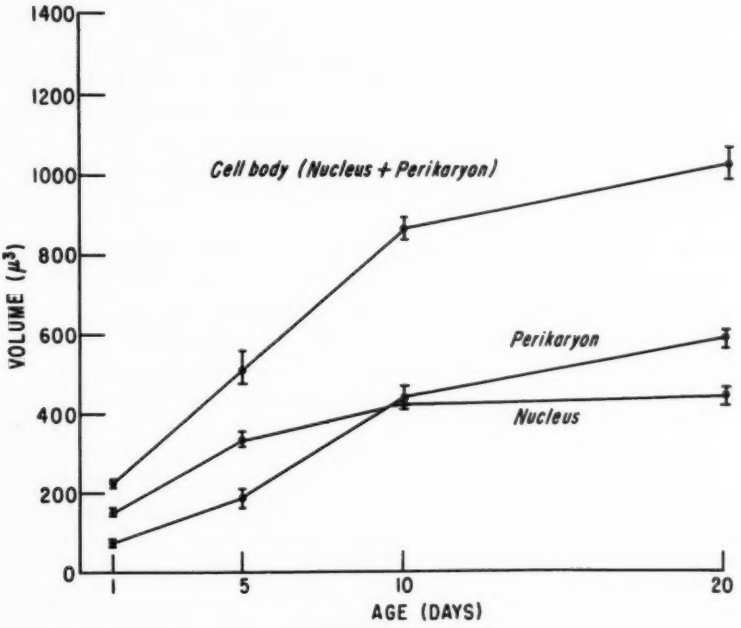

FIGURE 1
Postnatal changes in mean volumes of nucleus, perikaryon and total cell between 1st and 20th postnatal day.

The mean value for the nuclear/cytoplasmic ratio (Fig. 2) decreased from 2.12 at 1 day to 1.81 at 5 days, though it is clear from the magnitude of the standard errors as shown in Figure 2 that the difference is not statistically significant. The mean then decreased significantly to 0.95 at 10 days and to 0.75 at 20 days.

The gray/cell coefficient (Fig. 3) increased from a mean value of 6.54 at 1 day to 6.97 at 5 days and increased further to 8.00 at 10 days and 8.56 at 20 days.

Neuronal packing density (Fig. 4) decreased from a mean value of 680,000/mm^3 in the one day animals to 277,000/mm^3 at 5 days and decreased more slowly to 145,000/mm^3 at 10 days and 113,000/mm^3 in the 20 day group.

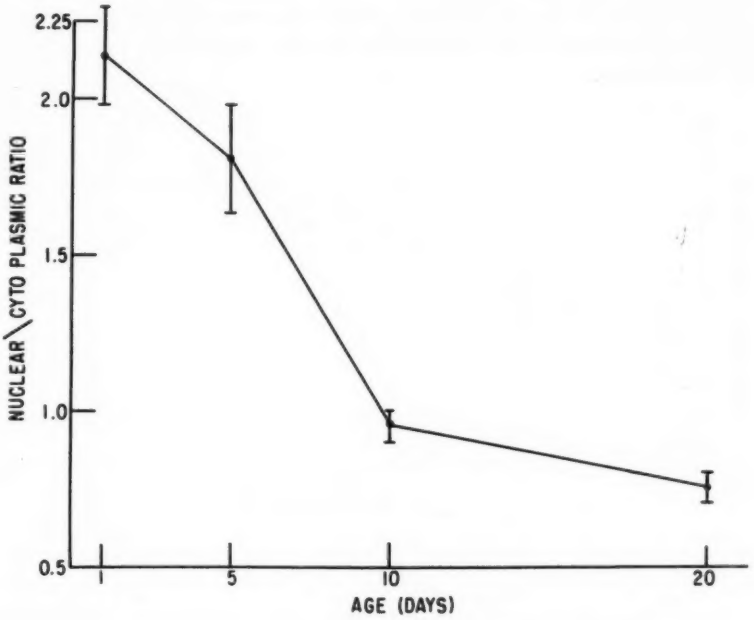

FIGURE 2
Changes in nuclear/cytoplasmic ratio in early postnatal stages.

Mean brain weight (Fig. 5) increased from a value of .33 gm in the one day groups to .55 at 5 days, .66 at 10 days, and attained a final value in the oldest groups studied of 1.26 gm.

Discussion. While the cerebral cortex in the rat at 20 days of age cannot be considered mature, the values for the various volumetric and cell packing density levels and relationships approach values found in mature rats. Neuronal packing density may be expected to decrease slightly in later stages while brain weight will increase to a normal adult level of about 2.0 gm. In preliminary studies in older age groups

(18) the mean perikaryon and nuclear volumes were found to decrease somewhat after the 20th day, then maintain fairly constant levels for a considerable time. These findings as well as Haddara's (2) (Table I and Fig. 2) on postnatal changes in perikaryal volume in the mouse appear to substantiate our earlier observations (17) in the posterior sigmoid gyrus in the cat where a similar decrease in mean perikaryon and nuclear volumes was found after an early rapid postnatal increase in these values.

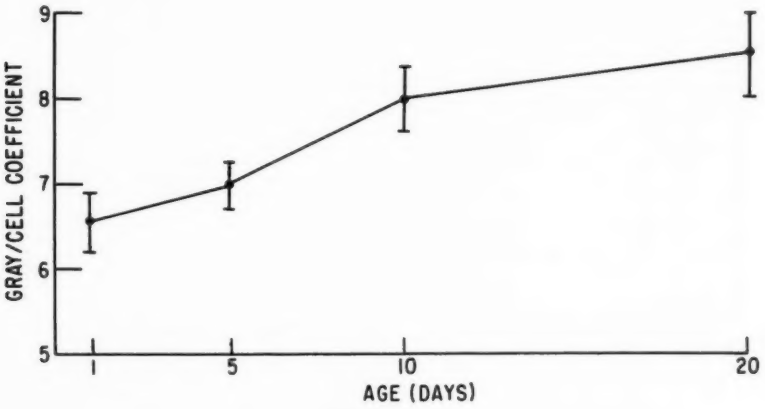

FIGURE 3
Increase in gray/cell coefficient in early postnatal stages.

It is of interest to note that Schadé (23), in a recent histological study of developing cerebral cortex, reported a cell body volume of 1156 μ^3 in 30 day old rabbits. This value is in good agreement with our value of 1031 μ^3 in the 20 day rat. Schadé, however, did not find any subsequent decrease in mean cell body volume as we have in the cat (16) and older stages in the rat (18) and as Haddara (22) showed in the visual cortex of the mouse. Further studies are needed to investigate this phenomenon in different species with a view to establishing its possible significance as a manifestation of basic metabolic changes during these early postnatal periods of development and maturation.

Quantitative histological determinations in the rat cortex can be

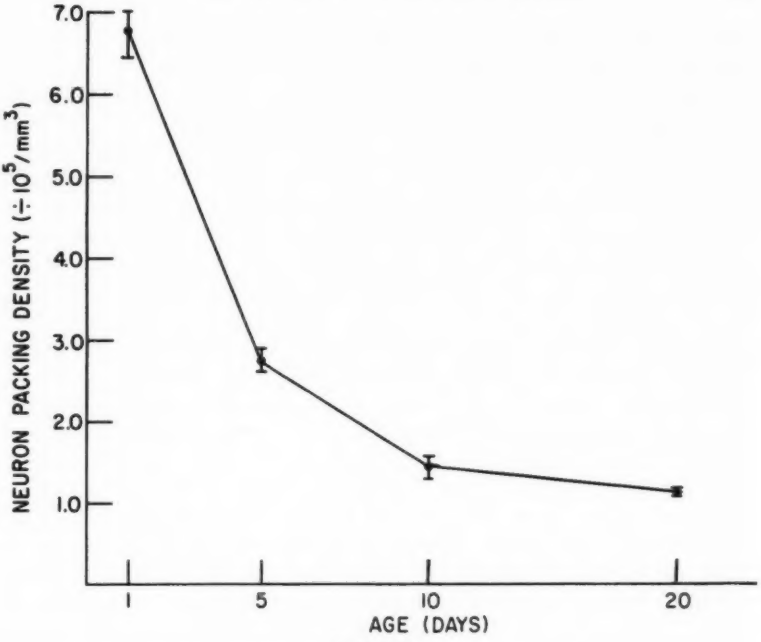

FIGURE 4
Early postnatal changes in neuron packing density.

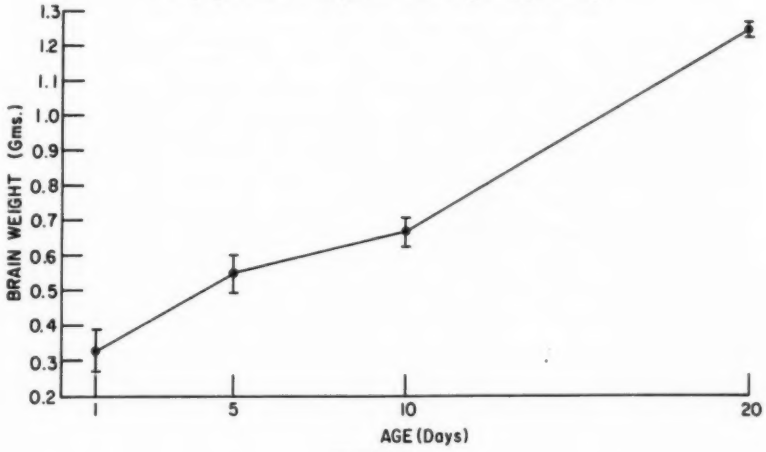

FIGURE 5
Increase in brain weight between 1st and 20th postnatal day.

carried out with somewhat less difficulty than in animals with well developed cortical gyri due to the fact that the surface of the cortex in the rat curves only slightly over the small area counted. In higher mammals the torsion produced in the formation of gyri tends to result in an increased packing of the cells in the deeper cortical layers underlying the crown of the gyrus. This condition tends to be more marked in gyri having more acute arches, as, for example, in the posterior sigmoid gyrus in the cat. This fact probably accounts for some of the discrepancies in quantitative histological data reported by various authors. Sholl (15), for example, reports neuron packing densities at 10 progressively deeper layers in the cruciate area of the cat cortex which when averaged give a packing density for the whole submolecular cortical thickness of 39,900 cells/mm^3. Our own mean value for the submolecular cortex of the crown of the posterior sigmoid gyrus in the motor area for the thigh in the cat (17) was considerably greater than this (67,000/mm^3) but our values in the outer layers are much closer to those reported by Sholl for the corresponding depths than in the inner layers. From this fact we assume Sholl's counts were made in a different part of the cruciate gyri than our own. This being the case, we believe the tendency toward packing of cells in the deeper cortical layers in the particular area we studied, resulting from the rather sharp arch of the gyrus at this point, probably accounts in large part for the observed discrepancy in results.

It is quite possible, however, that the counting method used by Sholl, employing the correction of Abercrombie (24), may result in somewhat lower counts than our own method even though we have taken into account the correction for nuclei bisected by *all* of the borders of the counting chamber. Abercrombie has pointed out that the greater the nuclear dimension in relation to section thickness, the greater the error in counting because of a greater proportion of nuclear fragments at the cut surfaces of the section. In our own studies and those of Peters and Flexner (9), the same principle should apply to *depth of focus* of the circle and to *diameter* of the circle or "counting chamber." In other words, the greater the volume of the "cylinder" or "counting chamber" delimited by focusing the circle through a fixed depth of tissue the lower the proportion of cells bisected by the borders of the chamber. In our method, however, we have adopted the procedure (16, 17) of counting all bisected nuclei in which more than half

of the nucleus is included in the circle, excluding those in which less than half is included in the circle and counting every other "half" nucleus. This procedure, employing the law of averages, should perform the same function as the correction of Abercrombie.

It is clear, however, that certain conditions should be met in order to assure the accuracy of this method. Consideration must be given to the space between the upper and lower borders ("roof" and "floor") of the counting chamber and the cut edges of the section. This space should be sufficiently deep to accommodate at least the radius of the lesser diameter of any nucleus in the section. If this condition is met there should be no question as to one's ability to evaluate any given nucleus bisected by the "roof" or "floor" of the chamber in terms of the proportion of it lying within the chamber. On the other hand, if the space between the upper and lower surfaces of the chamber and the cut surfaces of the section is too small to accommodate the radius of the lesser diameter of a nucleus, there might be some question as to whether or not the bisected nucleus in question should be counted.

In this connection it is important to note that the circle is focused in a plane perpendicular to the cut surfaces of the section and thus parallel to the plane of the outer or pial surface of the cortex. Since virtually all of the long axes of the neuronal nuclei are oriented perpendicular to the pial surface and parallel to the cut surfaces, our concern in providing sufficient space above and below our "counting chamber" is only with the lesser diameters of the nuclei.

Another consideration of importance is that of the diameter and depth of the chamber, or its total volume, relative to the greatest volume of the nuclei being counted. The chamber must be large enough to insure an accurate count but not so large that the investigator cannot keep track of all the nuclei included in the chamber and those bisected by its borders as he focuses through its total depth. As a result of our preliminary determinations of nuclear axis diameters and our experience with the Chalkley circle method in our preliminary studies (16, 17), we have adopted the criteria given in Table I for circle diameter and depth of focus which meet these requirements for each developmental stage studied.

In most quantitative histological studies of cerebral cortex in recent years and in modern neuroanatomy and histology textbooks as well, the term "perikaryon" has been employed to denote the neuron soma,

thus referring to the cytoplasmic body including the nucleus. In our studies in the field we have employed the term perikaryon in the sense in which it was originally employed, to indicate, as expressed in Dorland's Medical Dictionary (25) and in Herrick's textbook of neurology (26), that part of the cell cytoplasm which surrounds the nucleus but not including the nucleus.

Summary. Volume and density relationships were studied in four groups of rats varying in age from 1 to 20 days. Nuclear, perikaryon and total cell volume was found to increase significantly between the 1 and 10 day group but no significant differences were observed between the 10 and 20 day animals. The neuron packing density decreased rapidly between the first and tenth days and a slight decrease was observed between the tenth and twentieth days while the nucleo-cytoplasmic ratio decreased significantly between the first and tenth days, then maintained about the same level at 20 days. The gray/cell coefficient increased continuously from the first to the twentieth day as did brain weight.

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COMBINED INFLUENCE OF GRAVITY AND TEMPERATURE UPON GROWTH OF FRUIT FLY LARVAE*

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Over the past few years there have been several reports that increasing the effective gravity of an organism's environment can result in decreased growth. Mathews has demonstrated this with rats (3), Gray and Edwards with wheat (1, 2), Smith, Winget and Kelly with chickens and turkeys (6), and our laboratory with white mice (9) and fruit fly larvae (10, 11). This would be expected from physical considerations as reviewed by Thompson (8) and Rashevsky (4). The effect becomes more marked as the field increases. With larvae, the effect also becomes more marked as an animal's size increases. Although many factors in the environment are known to influence growth, gravity is probably the only environmental agent possessing the same constant intensity wherever normal growth is known to occur. Temperature, on the other hand, is highly variable.

The purpose of this paper is to report some observations on the interplay between these two factors in the control of physical development.

MATERIALS AND METHODS

Larvae of the common fruit fly (*Drosophila melanogaster*, wild, red eye) were employed because the larvae's rapid, exponential growth makes it ideal for mathematical analysis of development; also, it is possible to obtain and maintain large numbers of these animals with an economy of space. All observations reported here are on newly hatched animals during their first 24 hours of growth. Temperatures

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of 20°, 24°, 28°, 30°, 31.5°, 33°, and 36°C were employed. The animals were subjected to centrifugal fields which ranged in magnitude from 500 to 4000 times normal gravity. Admittedly, these magnitudes are much in excess of any gravitational field intensity which animals would normally encounter during their evolution or development on Earth. When one considers that acceleratory fields have a much more marked effect upon large bodies than small ones, these fields might be considered comparable in effect to normal gravity acting upon mammals.

Methods for culture and growth analysis did not differ markedly from previously described methods (10, 11). The larvae developed in plastic centrifuge tubes which contained a seed-free, banana-agar medium. For analysis of growth, the growth constant was defined as equal to the rate of change in the natural logarithm of volume. Geometric means of the volumes were employed. Volume was computed from measurements of length and width.

Increased gravity was simulated by centrifugation of the plastic tubes in Servall angle centrifuges (models SP or XL) at various speeds and distances from the center of rotation. The rotors and control tubes were enclosed in insulated chambers (see figure 1). These chambers were maintained at constant temperatures by means of Brownwill, constant-temperature circulators. Centrifuge tube holders and control tube holders both contained a small amount of water so that both atmospheres would be saturated. Both holders were dark inside.

The term G will be employed to refer to multiples of the Earth's gravity. The values following the $\pm$ sign refer to the standard errors.

Results

The ability of gravity to influence growth was greatly enhanced by raising the temperature beyond 20° C. No measurable effect could be demonstrated at 20° C (see figures 2 and 5).

At either normal or increased gravity a typical curve for rate versus temperature was obtained (see figure 3). The rate increased as temperature increased until an optimum temperature was attained, and then dropped off rapidly. The curves rise more slowly at the higher gravities. The activation energy between 20° and 28° is 12.2 $\pm$ 2.3 x 10^3 cal./mole at one G and 4.6 $\pm$ 1.7 x 10^3 cal./mole at

2800 G's. However, more observations are necessary in this temperature range in order to obtain reliable Arrhenius plots.

A drop in growth rates relative to control rates proceeds as the environmental temperature increases (see figure 4). As previously

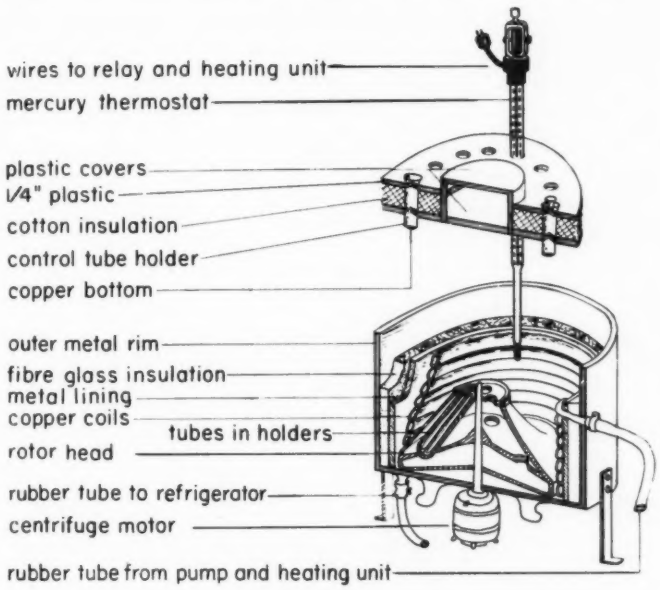
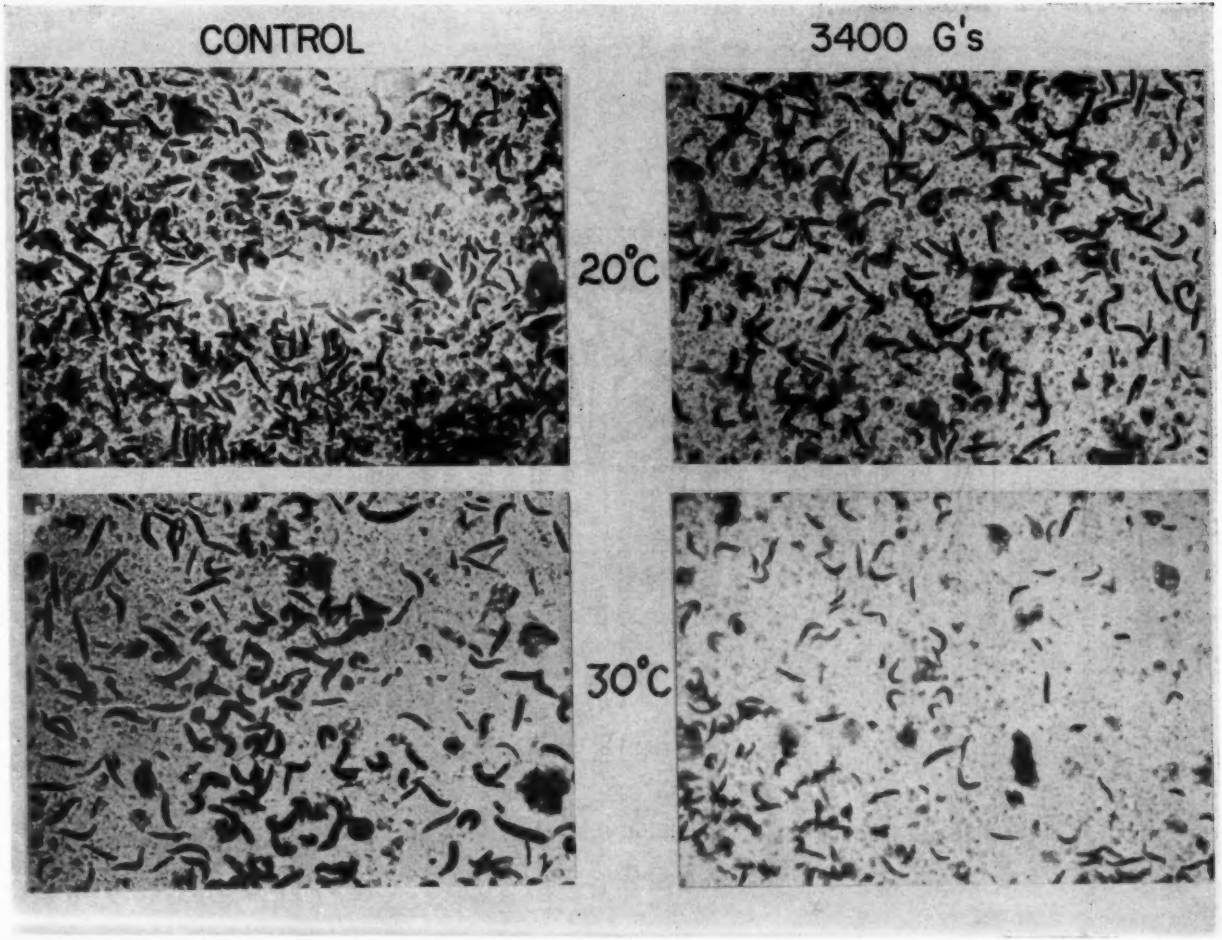


FIGURE 1

Constant Temperature Chamber for Centrifugation of Fly Larvae. Temperature of tubes in the control holders and centrifuge-tube holders agree within 0.1° C. The coils contain either water or an antifreeze solution and are placed in series with a Brownwill constant-temperature circulator and also, if necessary, with refrigerated coils. The mercury bulb from the circulating unit's thermostat dips into the chamber through holes in the plastic cover. Although the temperature in the chamber may oscillate by as much as 1° C, the average temperatures over 10-minute periods do not differ by more than 0.05° C.

mentioned, there is little effect at 20° C. However, at 31.5° the rate at 3000 G's is only one-half the control value. At 33° these experimental animals die before any growth occurs even though the controls demonstrate definite growth.

For temperatures at which gravity does evoke an effect, the growth constant drops in a more or less linear manner as the field increases


CONTROL
3400 G's
20°C
30°C

beyond 500 G's (see figure 5). Naturally, the slope for this drop is greater at higher temperatures.

Discussion

It was observed that altering both the temperature and gravity produces a much more pronounced effect than altering only one factor. Preliminary observations in our laboratory indicate that the same phenomenon occurs with centrifuged mice. Although we are unable to explain why centrifugation is capable of limiting the growth

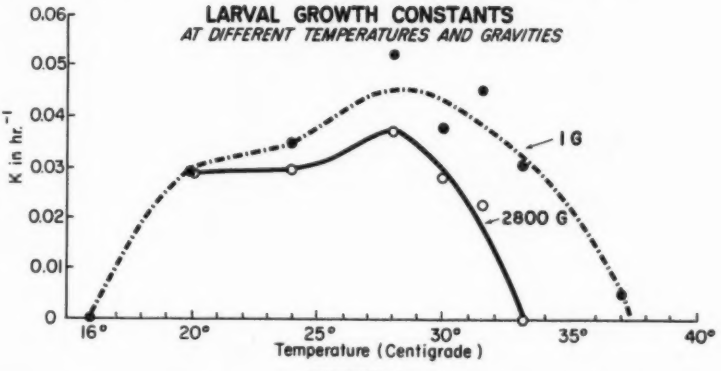


FIGURE 3

Growth Constant (k) as a Function of Temperature at Two Different Gravities. The experimental k's at 28° C and at 31.5° C were computed from the best fitting straight lines in figure 5.

rate more readily at higher temperatures, there are four alternate suggestions as to what the mechanism involved might be.

1. *Interference with Some Active Process.* For the temperature range between 20° and 28° C, the controls exhibit an activation energy (12,000 cal./mole) which would be expected for an enzymatically catalyzed process. The larvae during centrifugation at 2800 G's display an activation energy (4600 cal./mole) close to that of viscosity-dependent processes. Perhaps some enzyme which enhances growth at higher temperatures is not produced. The sites for producing the enzyme might be destroyed by centrifugation. If this enzyme is not absolutely essential for growth but merely speeds up some part of the process, the organism may, as an adaptation to high gravity, cease

production of the enzyme. The food materials which would normally be used for producing this substance could be diverted to be employed directly in a slower but more efficient growth.

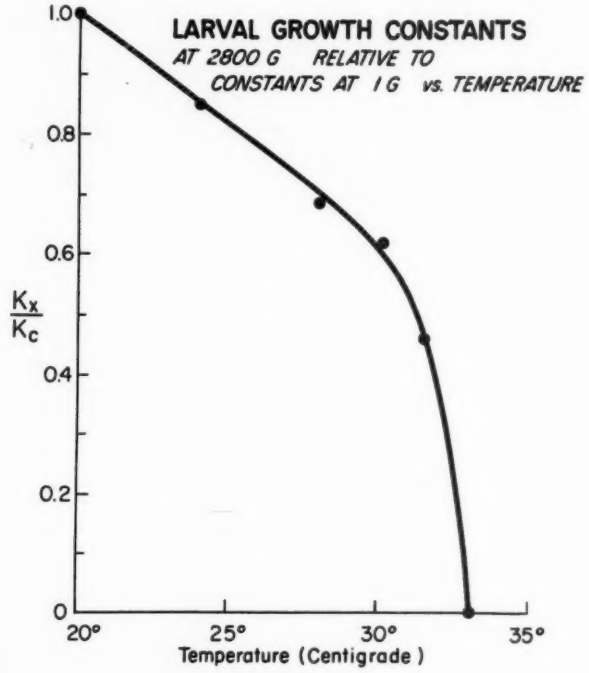


FIGURE 4

Decrease in "Relative" Growth Constant (k_x/k_c) as a Function of Temperature at 2800 G's. These values can be obtained by dividing the value for k at 2800 G's by the control vaue for k at the same temperature.

2. *An Optimum Temperature for Adaptation to Gravity.* Suppose the optimum temperature at which these animals could adapt in such a way as to increase the efficiency of growth were at 20° C. Then an increase in temperature would result in a slower growth rate relative to the control rate.

3. *Altering the Temperature Regulating Ability.* Although insects are not warm-blooded animals, they can sometimes maintain them-

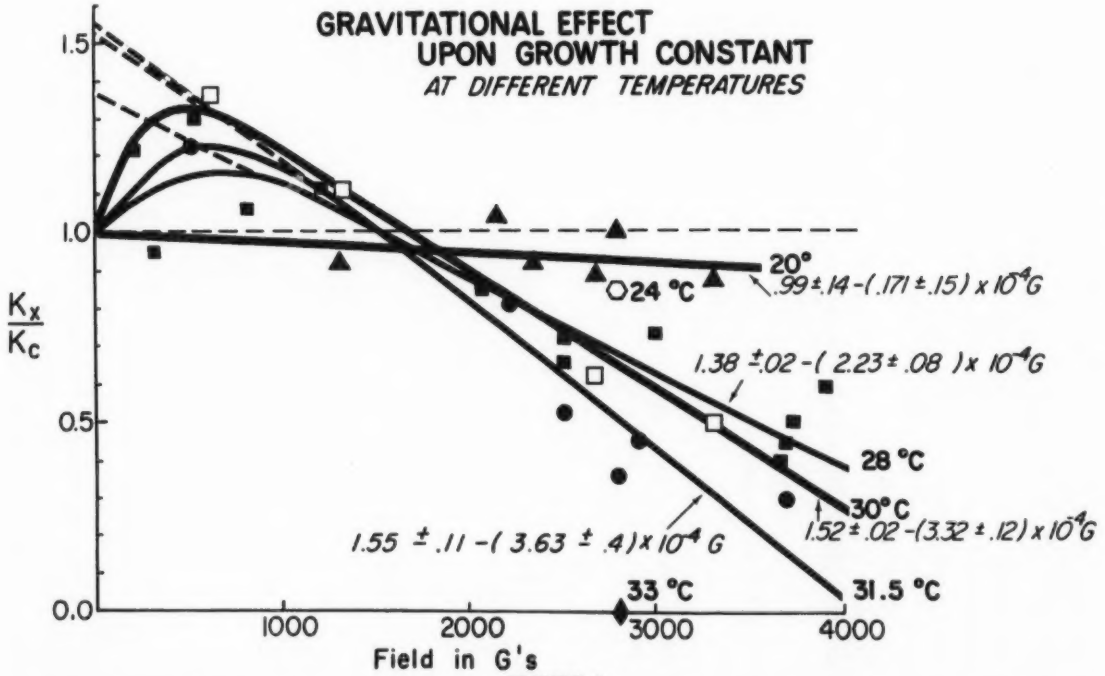


FIGURE 5

"Relative" Growth Constants (k_x/k_c) as a Function of Gravitational Field at Different Temperatures. Each point represents measurements from approximately 60 experimental and 60 control animals. The errors for each point are equal to approximately 10 per cent of the magnitude represented by the individual experimental points.

selves at temperatures which are intermediate between that of the environment and that best suited for the organism (5). High gravity could cause less energy from food materials to be available for temperature regulation. The control animals would keep themselves at a more ideal temperature. Let us assume that 20° is close to the animal's ideal temperature. At higher environmental temperatures, more temperature regulation would be necessary and centrifugation would have a greater effect.

4. *A Temperature-Dependent Sedimentation of Some Material.* Perhaps an unequal distribution of this material is required to trigger an adaptation to gravity. At appropriate temperatures, high gravities can cause concentration gradients as a result of sedimentation. With higher temperatures, the greater Brownian motion of particles would tend to overcome the effect of sedimentation. For a discussion of the physical theory behind such conditions of "sedimentation equilibrium" the reader is referred to the work of Svedberg and Pederson (7).

All of these suggestions are related to the concept that larvae undergo adaptation to increased gravity. As the gravity increases, more food materials would be diverted from growth or growth-related processes in order to work against gravity. Also, high gravity could reduce food consumption as is the case with mice. There is evidence that these larvae can adapt to gravity and grow in a more efficient manner (Wunder, Herrin, and Cogswell, 11), thus partially compensating for the proposed diversion of food materials. After centrifugation, the animals require less oxygen for the same amount of growth and under certain conditions can even grow at faster rates than control animals. During exposure to low centrifugal fields the larvae can even grow faster than the controls. With experimental larvae of the same age and size those placed in the centrifuge at the youngest age grew at a faster rate. This would indicate that the larvae were better able to adapt if placed in the centrifuge when as young as possible. Most larvae which were removed from the centrifuge would, like the controls, develop into pupae. However, unlike the controls, very few attained the adult stage. This suggests that, in adapting to centrifugation, the organism for the sake of immediate efficiency sacrifices tissues or organs which were not required until the onset of the developmental rearrangements associated with the pupal stage. Any way in which temperature could alter an adaptation would in turn alter the response to centrifugation.

SUMMARY

Increased gravity can reduce the growth rate of Drosophila larvae. With newly hatched larvae, this reduction becomes more marked as the environmental temperature rises above 20° C. The reason for this interplay between these two physical factors is unknown. Four tentative explanations are suggested to account for this phenomenon. These alternate explanations are related to the animal's adaptation to centrifugal fields.

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